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THE ROLE OF I^{131}-LABELED PROTEINS IN BIOLOGY AND MEDICINE

BY

S. P. MASOUREDIS (*Conference Chairman*), F. E. BEACH, S. A. BERSON, F. J. DIXON, A. L. FLICK, D. GITLIN, P. D. GOLDSWORTHY, W. L. HUGHES, N. D. LEE, A. S. MCFARLANE, S. MARGEN, R. A. MILCH, R. R. PATON, D. PRESSMAN, E. B. REEVE, J. L. STEINFELD, D. L. TABERN, D. W. TALMAGE, H. TARVER, W. VOLWILER, W. O. WEIGLE, AND R. S. YALOW

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THE ROLE OF I^{131}-LABELED PROTEINS IN BIOLOGY AND MEDICINE*

Conference Chairman and Consulting Editor
S. P. MASOUREDIS

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Part I. Methodology

Part II. Applications

* This series of papers is the result of a conference on *The Role of I^{131}-Labeled Proteins in Biology and Medicine* held by the Section of Biology of The New York Academy of Sciences, November 30, 1956. The conference itself was aided in part by grants from Abbott Laboratories, North Chicago, Ill., and Eli Lilly and Company, Indianapolis, Ind.

Part I. Methodology

THE CHEMISTRY OF IODINATION*

By Walter L. Hughes

Medical Department, Brookhaven National Laboratory, Upton, N. Y.

Introduction

The reactions of proteins with iodine probably have received more study than those with any other reagent. The reasons for this become obvious when one considers the simplicity with which these reactions may be accomplished, the readiness with which their products can be analyzed, and the striking hormonal properties of some of the heavily iodinated derivatives. The complex pathways of iodination thus revealed may at first dissuade one who is searching for a reliable method of labeling proteins. However, further consideration suggests that a simple solution is perhaps impossible to achieve in structures as complicated as proteins, and a similar complexity also may develop with other reactions of proteins when they are investigated in equal detail. From this point of view, iodine has real merit as a label because of the wealth of information concerning it.

Prerequisite to an understanding of the reactions of iodine with proteins is some knowledge of its interactions with the solvent. The most important of these are listed in TABLE 1. Since iodine is soluble only to the

TABLE 1

EQUILIBRIA OF IODINE WITH SOLVENTS*

$I_3^- \rightleftarrows I_2 + I^-$	$K = 0.0013$	(1)
$I_2 + H_2O \rightleftarrows H_2OI^+ + I^-$	$K = 1.2 \times 10^{-11}$	(2)
$I_2 + OH^- \rightleftarrows IOH + I^-$	$K = 30$	(3)
$HOI \rightleftarrows H^+ + OI^-$	$K = 10^{-11}$	(4)
$3OI^- \rightarrow IO_3^- + 2I^-$		(5)
$I_2 + RNH_2 \rightleftarrows RNHI + H^+ + I^-$		(6)

* Equilibrium constants for REACTIONS 1, 3, and 4 are taken from Latimer;[1] that for REACTION 2 is from Bell and Gellis.[2]

extent of 1.1 mMol/liter in water at 20° C., some agent such as iodide, which forms soluble complexes, usually is added. The resulting equilibrium (REACTION 1, TABLE 1) has two important additional effects: (1) it slows the reaction rate by decreasing the concentration (activity) of iodine; (2) it "buffers" the reactivity. Iodide ion normally is produced during the reaction with protein, and therefore an excess of I^- minimizes the changes in activity during reaction.

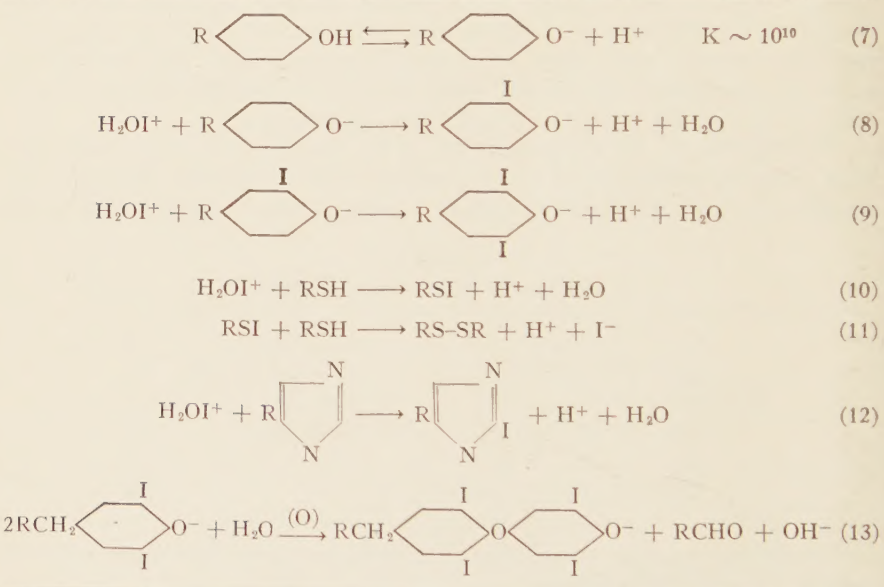
* The work reported in this paper was supported by the United States Atomic Energy Commission, Washington, D. C.

REACTION 2 (TABLE 1) indicates that the ionization of iodine gives the active species, H_2OI^+. Since another iodide ion is produced, the rate of reactions involving this species now must vary inversely as the square of the concentration of iodide.

REACTION 3 (TABLE 1) shows the hydrolysis of I_2 to HOI. REACTION 4 (TABLE 1) indicates the ionization of hypoiodous acid ($pK = 11$). REACTION 5 (TABLE 1) indicates an adverse sequel to this ionization: the irreversible formation of iodate, which sets an upper practical limit[3] for iodination of pH 10 (fortunately well above the stability limits of many proteins). REACTION 6 (TABLE 1) indicates a competing reversible process that may take place with ammonia or with any amino group and, probably, with other nitrogenous bases, including those of the protein. Like the first equilibrium, this should serve only to depress the rate of reaction, without altering the ultimate result.

TABLE 2

REACTIONS OF IODINE WITH PROTEINS



Reactions with Proteins

TABLE 2 shows the principal reactions of iodine with proteins. These processes are either irreversible (or essentially so) in that the equilibrium is far to the right, as in the case of SH oxidation. The reactions that involve substitution into the tyrosyl residues have been written first, since they are probably the basis of all tagging with radioactive iodine. The reactions with sulfhydryl always occur much more rapidly; however, they do not result in stable bonding of iodine. Whenever geometrical factors permit,

REACTION 11 (TABLE 2) takes place, liberating all of the iodine as iodide ion. In the case of monothiol proteins such as serum albumin, in which the formation of a disulfide link is sterically difficult, two equivalents of iodine are consumed, but the sulfenyl iodide (REACTION 10) must hydrolyze rapidly, since the final product is devoid of iodine[3] (TABLE 4).* Consequently, in any labeling experiment the first iodine consumed, equivalent to the SH content of the solution, must be considered lost for purposes of labeling.

Before considering iodination of tyrosine in detail, I shall discuss briefly the other known side reactions: 12 and 13 (TABLE 2). Both of these occur appreciably only upon the introduction of large amounts of iodine into the protein molecule, and thus are probably not important at "tagging" levels.

Iodinated derivatives of histidine that contain iodine on either C or N of the imidazole ring[5] have been prepared. However, only carbon-bound iodine is stable to sulfite. Li[6] has measured the rate of iodination of histidine, and he has shown that it is the only other amino acid likely to be substituted under the usual iodinating conditions, although tryptophan may be destroyed.[3, 7] Evidence for the iodination of histidyl residues in proteins is based upon analogy with the free amino acids coupled with the finding of bound iodine in excess of the sites available in tyrosyl residues.[3, 8] To my knowledge, iodinated histidine has not been isolated from a digest of the iodinated protein. Tyrosyl residues usually iodinate more readily than histidyl, but the difference is not extreme. Thus, in iodinating serum albumin, the less reactive portion of the tyrosyl residues reacted concomitantly with histidyl.[3]

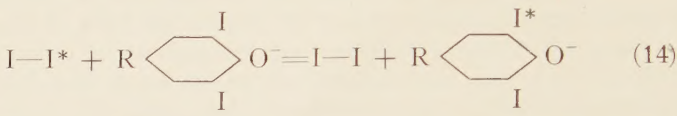
Thyroxine formation (REACTION 13, TABLE 2) also occurs in the later stages of iodination. While the mechanism of the reaction is not clear, the equation has been written to imply certain aspects of the reaction, such as its occurrence at a high content of diiodotyrosine and in an oxidizing milieu (excess iodine). The yield varies from protein to protein and may reach 20 per cent of the theoretical (based upon tyrosine content) in exceptional cases. This phase of iodination has been reviewed recently by Roche and Michel.[9]

Returning to the reaction with tyrosine which, as already stated, is the basis of iodine tagging, REACTIONS 8 and 9 (TABLE 2) indicate that the reaction takes place in two stages. The reaction has been written in this form to imply an ionic mechanism between cationic iodine and the anionic phenolate ion.† Such a mechanism best fits the experimental evidence for iodination in acid solutions.[10] Whether it can be extended to iodinating conditions above *p*H 7, where other species such as hypoiodous acid become much more abundant is, of course, conjectural. However, this mechanism agrees well with the inverse dependence of the reaction rate on the hydrogen ion

* Fraenkel-Conrat has recently found an exception in the case of tobacco mosaic virus.[4] This molecule apparently forms a stable sulfenyl iodide in which the iodide is not reactive toward reducing agents and does not exchange with free iodide ion. However, it is liberated upon denaturation of the protein.

† Probably as its quinoid form

concentration and on the square of the iodide ion concentration.[11] Furthermore, the introduction of the second iodine atom takes place more readily than the first. This also may be explained by assuming that reaction involves the tyrosylate ion, since iodination increases the acidity of the phenolic hydroxyl, thereby increasing the abundance of the substituted phenolate ion correspondingly.* A similar mechanism may be involved in the rapid exchange observed between iodine and diiodotyrosine:



(14)

This reaction, the basis of present methods for the preparation of radioactive diiodotyrosine, is unique for phenols—even phenol ethers do not react.[13, 14] The rate of this exchange reaction also varies inversely as the H^+ concentration; its dependence on I^- concentration was not studied.

The kinetics of iodination by I_3^- have been studied by Li both for free tyrosine[11] and for serum albumin[15] (FIGURE 1). The conditions used (*p*H 5 to 6 at 25° C.), while undesirable for labeling most proteins, were chosen for better rate measurements.† However, the principles thus established seem capable of extension to the practical region above *p*H 7. Li observed a marked variation in the reactivity of the tyrosyl residues of serum albumin. In fact, his rate curves appeared to be extrapolating to a value corresponding to iodination of two thirds of the tyrosyl residues. As already noted, we have also found a variation in reactivity, but we find that complete substitution of tyrosyl can be accomplished, although accompanied by considerable iodination of histidyl residues.[3]

Heterogeneity related to the site of labeling becomes a new variable to consider in interpreting results with labeled proteins. Since labeling is used to trace unlabeled protein, it must be assumed that labeling does not alter the properties of the protein so identified, but proof of this implies a direct comparison with the unlabeled protein—obviously a circuitous proposition. Nevertheless, several precautions can be taken. Perhaps the best of these is a comparison of products labeled to different extents where, if identical results are obtained, an extrapolation to the unaltered protein would seem reasonable. This hypothesis is, of course, based on a progressive change in properties with increasing iodination such as would be expected in the instance of a protein that contained a large number of groups per molecule with similar reactivities. Obviously, this reasoning fails completely in the case of a functional group such as sulfhydryl, which is inactivated completely by the first increment of iodine added.

Assuming identical sites that do not interact with each other, the distri-

* Roche, Lissitzky, and Michel[12a] and Gemmill[12b] presumably have prevented this acceleration in the preparation of monoiodotyrosine by carrying out the reaction in concentrated ammonia or in dilute KOH, above the *p*K of tyrosine itself.

† Herriott labeled pepsin successfully at *p*H 5 to 6, since it is not stable at higher *p*H values.[16]

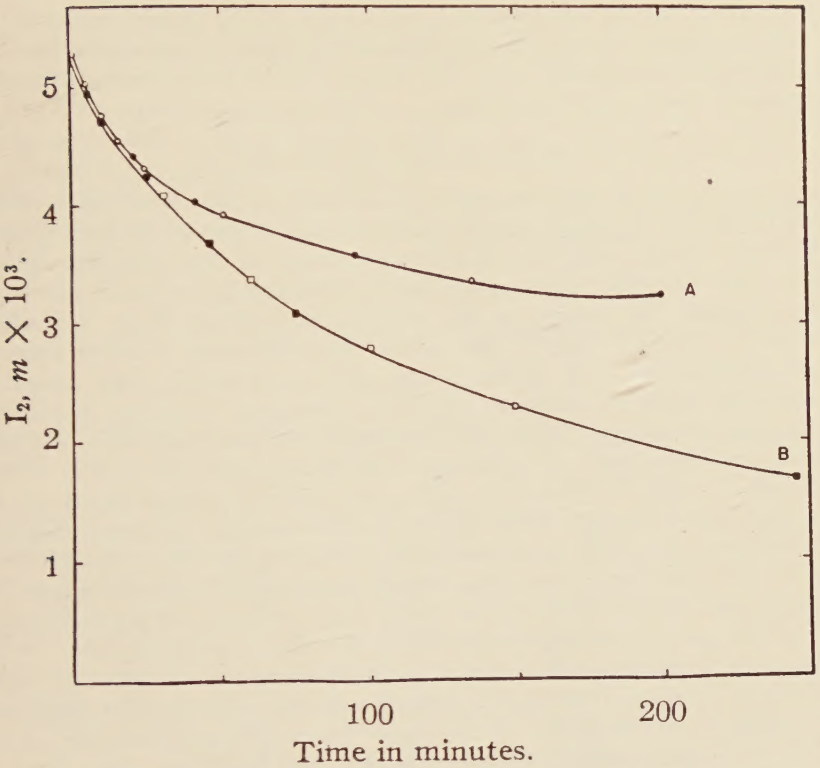


FIGURE 1. The rate of iodination of human serum albumin (curve A) and of tyrosine (curve B) in *p*H 5.7 acetate buffer at 25° C. Serum albumin concentration is 12.5 gm./.; tyrosine concentration is 2.65×10^{-3} *M*; iodide concentration is 3.4×10^{-2} *M*. The initial concentration of iodine is stoichiometric for double substitution in tyrosyl residues. Reproduced by permission from *The Journal of the American Chemical Society*.[15]

bution of label at any level of iodination can be predicted from the laws of probability.[34] The greatest homogeneity, with respect to amount of label, will occur at the beginning and at the end of the reaction. Only two species occur at the beginning: those with no iodine and those with one atom; and, at the end, those with "*n*" iodine atoms and those with $n - 1$. (Of course, if *n* represents the number of iodine atoms in tyrosyl, increasing homogeneity with respect to these may be accompanied by increasing heterogeneity relative to histidyl substitution.)

Consequently, minimal labeling would seem desirable. However, if only a small fraction of the molecules is labeled, one has the additional concern as to whether one is labeling an impurity. Therefore, perhaps an average of 1 atom of iodine per molecule of protein (or 2 atoms, to allow for the formation of diiodotyrosine) seems desirable. In the latter case, assuming monoiodotyrosine formation on identical sites, most of the label still will be found on species that contain 1 to 4 iodine atoms, and only a small fraction of

uniodinated proteins will remain. Even in this case it should be noted that if any inactivation occurs, it will represent a larger percentage of the more heavily iodinated species; thus, as always, tracer experiments will overaccentuate the inactive component.

Other Methods of Labeling

Iodo compounds may be employed to label proteins that lack tyrosine, such as gelatin, or where direct iodination leads to loss of function. In general, such proteins make use of iodine previously incorporated into an aromatic ring (aliphatic iodine is too labile) that contains another functional group suitable for binding to the protein: IϕX, where X = $-SO_2Cl$,[17] $-COCl$, $-NCO$,[18] $-N_2^+$,[19] and so on. The first three of these reagents react preferentially with amino groups and, consequently, they should be particularly useful in instances where the iodination of tyrosyl residues is destructive of function. These iodo compounds will also react with sulfhydryl groups and thus show no advantage over direct iodination in this regard, although it might be easier to protect the sulfhydryls from attack as, for example, with CH_3Hg^+. These labels possess the added advantage of iodine so bound that it cannot enter the iodide pool even after the degradation of the protein. This may prove useful where corrections for thyroid uptake are difficult, and it may even provide more rapid excretion of degraded label.

In the case of *p*-iodobenzenesulfonyl chloride (pipsyl chloride),[17b] the label is so stable that it has proved useful in structural studies since, upon hydrolysis, it always remains conjugated with the reacted amino acid, thus permitting a determination of the immediate amino acid sequence at the point of its attachment.[17a] Pipsyl chloride's chief limitation stems from its insolubility in water, which necessitates coupling to the protein by mixing it in organic solution (dioxane is quite satisfactory) with the aqueous protein solution buffered above neutrality. Under these conditions it is difficult to prevent precipitation of both the protein and the reagent and, in addition, the danger of denaturation is relatively great.

The isocyanates, including their halogen derivatives, have been studied extensively as labeling reagents.[18] However, these compounds are quite as insoluble as the sulfonyl chlorides and, in addition, they produce symmetrical diphenyl ureas as by-products that also are insoluble and difficult to remove. Nevertheless, Hunter[20] has recently found *p*-iodophenyl isocyanate (or the isothiocyanate) particularly useful for labeling insulin since it reacts preferentially with the α-amino groups and a homogeneous monosubstituted derivative can be isolated.

Some of these reagents are at least tedious to synthesize and, since I^{131} has a half life of only eight days, their routine use would require repetitive chemistry every few weeks. Obviously, the ideal reagent does not exist, but certain specifications for such a reagent may be noted here in the hope of stimulating further research:

(1) Radioiodine should be introduced as late in the synthetic process as possible—preferably in the last step, so as to minimize the amount of repetitive chemistry.

(2) The reagent should be a relatively small, water-soluble molecule that will react with protein within 2 pH units of neutrality at room temperature or lower.

(3) The derivative should be as stable as the protein, and the iodine should be in stable linkage and, preferably, not capable of dissociating to free iodide in any physiological process.

(4) The radioactive products of degradation should not be reutilized by the organism and should be excreted as rapidly as possible.

Isotopes of Iodine

Several isotopes of iodine are available for labeling purposes (TABLE 3).

TABLE 3
USEFUL RADIOISOTOPES OF IODINE

	130	131	132	133
Half life	12.6 hr.	8.05 days	2.33 hr.	21 hr.
β's (Mev)	1.02 0.60	0.608 0.335 0.250	2.12 1.53 1.16 0.9	1.3 0.4
γ's (Mev)	0.74 0.66 0.53 0.41	0.722 0.637 0.364 0.284 0.080	2.2 1.96 1.40 1.16 0.96 0 777 0.673 0.624 0.528	1.4 0.85 0.53
Source	Cyclotron	Oak Ridge	BNL*	BNL*

* Brookhaven National Laboratory.

The early studies of radioactivity that used accelerator-produced isotopes of iodine have been reviewed by Kamen.[21] Today the uranium fission product, I^{131}, is used almost exclusively.* I^{131} appears to be "built-to-order" for many purposes. Its 8-day half life is long enough for complex chemical manipulation, and yet it is short enough to exclude long-term radioactive contamination. It emits γ rays that permit simple counting procedures without chemical preparation of the sample, and β rays that provide localized irradiation of the particular tissue (for example, the thyroid) in which it is located.

For special applications, isotopes of different half lives or with different radiation properties may be preferred. No iodine isotope of usefully longer

* Obtained carrier-free from Oak Ridge National Laboratory, Oak Ridge, Tenn.

half life exists, but two with shorter half lives, I^{132} (2.3 hr.) and I^{133} (21 hr.) are now available.* These radioisotopes possess radiation spectra quite similar to I^{131}, permitting measurement with the same counting equipment. However, the shorter half lives permit repetitive experiments to be carried out on the same individual, allowing a few half lives for decay. Also, experiments with double labels can be performed, the two activities being determined by counting each sample twice: immediately, and after a suitable interval to allow the first isotope to decay. Shorter half lives permit much larger tracer doses to be administered without radiological effects on the individual, since the cumulative dose is inversely proportional to the half life, assuming a constant concentration in the tissue.

I^{132} is supplied in a convenient form[22] as its 77-hour half-life parent, Te^{132}. The container has been designed so that the iodine can be extracted conveniently from the tellurium as needed, whereupon the tellurium regenerates a new equilibrium amount of I^{132}. This isotope has been used largely as iodide, although a study has appeared in which it was used to label serum albumin.[23]

Isotopes of iodine with very different radiation properties exist, but thus far have not been developed. Perhaps most intriguing of these are the positron emitters I^{124} and I^{126}, which can be prepared in cyclotrons. Positron emission is currently receiving much interest because of the unique annihilation radiation: two hard γ rays recoil in opposite directions when the positron meets an electron. Much more precise localization of the point of origin of the radiation then may be obtained with the aid of detectors on opposite sides of the body so triggered electronically as to record only the events that they see simultaneously.[24, 25]

No isotope of iodine emits α particles. However, astatine, element 85, the last of the halogens, possesses an isotope At^{211} that is an essentially pure α emitter (FIGURE 2). At^{211}, with a 7.3-hour half life, is conveniently prepared from bismuth in the cyclotron. Some preliminary studies indicate that astatine can be incorporated in proteins.[27]

The unique potentialities of α-rays stem from their very short range, which permits their microscopic localization, and their high rate of linear energy transfer, which results in tracks in photographic emulsion. These tracks are straight and of constant length, so that it becomes possible to locate the point of origin of α-emission with high precision by autoradiography.

Properties of Labeled Proteins

The stability of iodine substituted in tyrosine appears sufficient for biological purposes. However, this is not true of some other substitutions (see REACTIONS 5 and 10, TABLE 1). Nevertheless, iodine may be bound tightly enough to other structures in proteins to require chemical procedures for its removal. Thus, *N*-iodo bonds are not easily split by dialysis, but they respond readily to reduction by bisulfite; after this treatment the iodide may be removed by dialysis.

* Obtained from Brookhaven National Laboratory, Upton, N. Y.

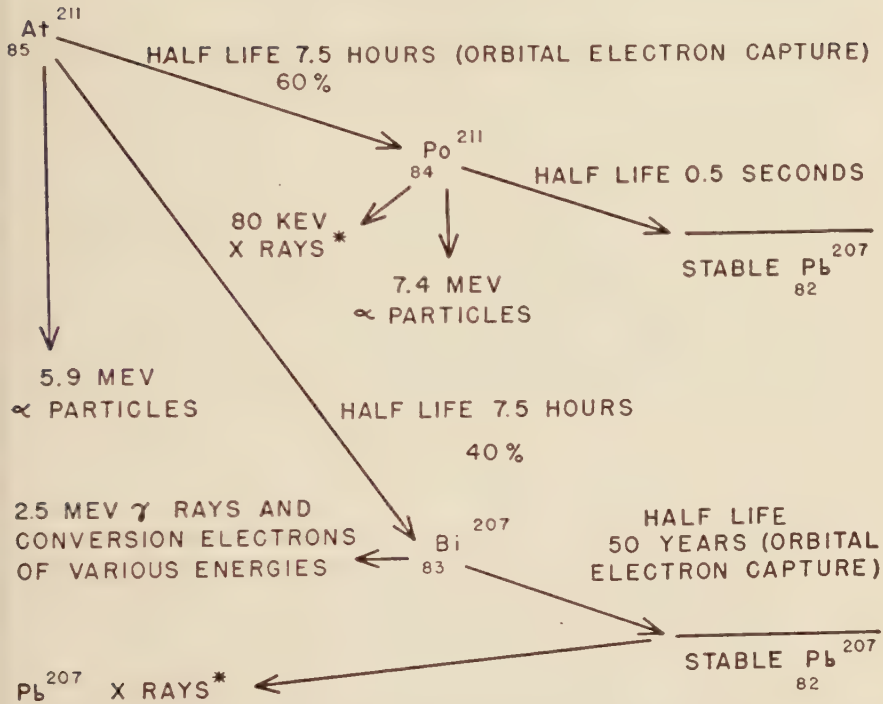


FIGURE 2. Decay scheme of astatine[211]. Note that 60 per cent produces an α particle after a one-half-second "stopover" as Po^{211}. The 80 Kev X rays are ideal for counting in a crystal scintillator. The 50-year half-life daughter, Bi^{207}, does not interfere for most purposes. Reproduced by permission from the *University of California Publications in Pharmacology*.[28]

The iodophenol bond is labile in strong acids; therefore, hydrolysis of iodinated proteins is normally carried out in alkali. Also, as pointed out above, iodophenols exchange readily with free iodine above *p*H 3 and, consequently, under somewhat physiological conditions. However, no evidence of this has been observed *in vivo* and, since the mechanism requires oxidized iodine (iodide is inactive[13]), its occurrence seems unlikely at the low oxidation-reduction potentials found *in vivo*.* Furthermore, heterologous labled albumin disappears from the blood at the same rate whether measured immunochemically or radioactively. In a study that possibly was more rigorous, Lewallen and I have recently looked for exchange by injecting cold labeled human serum albumin into a dog to which radioiodide had been administered.

"The dog was placed on a 10% glucose diet for 3 days before the experiment to restrict iodide excretion, then given 2 mc. of carrier-free I^{131} and

* The thyroid is known to oxidize iodide. Could exchange into thyroglobulin account for some of the thyroid uptake of radioisotope?

0.4 gm. of iodinated human serum albumin, containing 35 atoms of I^{127} per molecule of protein. Thus the dog was given several times its body pool of iodine as iodinated albumin. Blood samples were removed on successive days, centrifuged, and the human serum albumin precipitated by rabbit antiserum and thoroughly washed. No detectable activity was ever found in the specific precipitate (i.e., less than 0.01% of the total activity of serum)."[28]

Physicochemical changes upon iodination vary, of course, with the protein and with the degree of iodination. The absorption spectra show changes characteristic of the iodination of tyrosine with the appearance of a new maximum at 305 to 315 mμ, depending on the *p*H, since the phenolic hydroxyls of monoiodotyrosine and diiodotyrosine have *p*K's of 8.2 and 6.5, respectively.[30, 33] FIGURE 3 shows typical absorption spectra of several iodinated albumins at *p*H 10.7. This *p*H was chosen as intermediate between the *p*K of the tyrosyl and diiodotyrosyl residues, so that a maximum shift in spectrum would follow iodination. A shift in the maximum toward lower

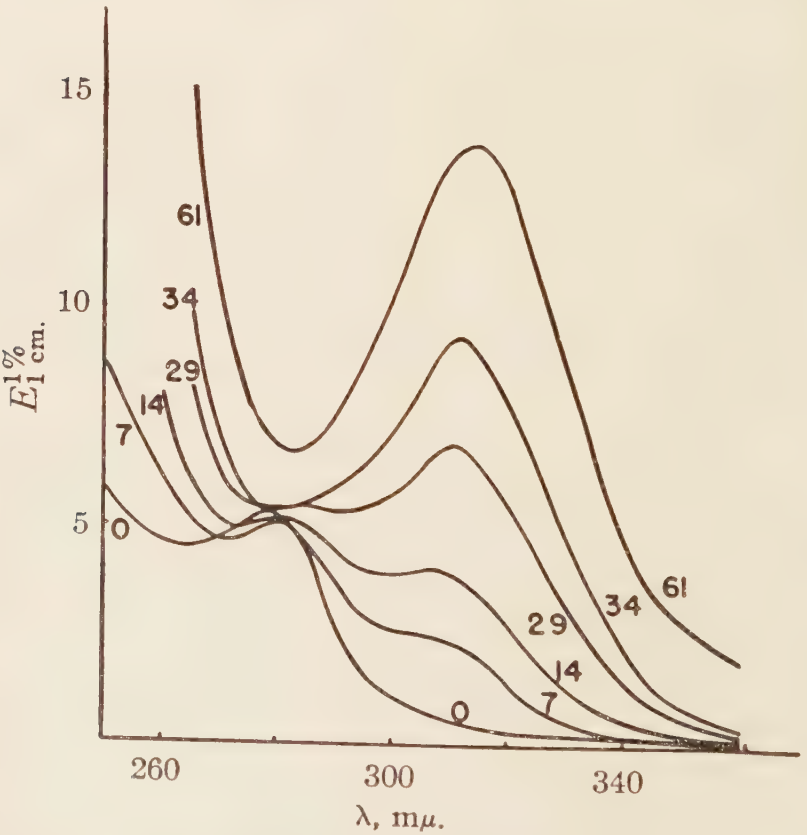


FIGURE 3. Absorption spectra of iodinated human serum albumins at *p*H 10.75. The numbers on the curves indicate the atoms of iodine per molecule of protein. Reproduced by permission from *The Journal of the American Chemical Society*.[3]

wave lengths with small degrees of iodination suggests the formation of monoiodotyrosine, which has an absorption maximum of 305 mμ at this *p*H. It may be further noted that the diiodotyrosine peak does not reach a maximum when the stoichiometric amount (30 atoms/molecule) of iodine is introduced.

Iodination progressively decreased the solubility of serum albumin to the point at which albumin containing 34 atoms of iodine was insoluble in distilled water.[3] Similar results were obtained with pepsin by Herriott, who further found that iodinated pepsin no longer showed constant solubility with excess saturating body.[29] This finding was to be anticipated from the known heterogeneity with respect to label, since only partially iodinated products could be crystallized for analyses of solubility.

Herriott observed no change in the electrophoretic mobility of iodinated pepsin at *p*H 4.[16] However, at higher *p*H values, an increased mobility should be found where the diiodotyrosyl residues are ionized. An increase has, in fact, been observed with commercial radioiodinated serum albumin at *p*H 8.6.[31]

Neuberger[32] found that Iodozein showed change in its titration curve quantitatively attributable to its content of diiodotyrosine: the curve, normally almost flat between *p*H 8 and 11 (in alcohol), upon iodination becomes very steep and shows an additional 30 titrating groups per 100,000 gm. This number corresponds very well with analytical data that show 32 mols of tyrosine per 100,000 gm. Similar changes in the titration curve have been observed in iodinated pepsin and serum albumin, although the changes were not as interpretable quantitatively in terms of the content of diiodotyrosine. Pepsin has 16 tyrosyl residues, and a product that contained 43 iodine atoms required 20 more mols of alkali per mol of protein to raise the *p*H from 6 to 10 than did uniodinated pepsin.[16] Herriott suggests the value 20 may be too high due to electrolytic reduction at the hydrogen electrodes with the formation of hydriodic acid, since he observed dialysable iodine after the titration of his samples. Serum albumin gave an even more confusing picture, no net increase in the number of titrating groups being observed in the region where diiodotyrosine residues were dissociating.[33] In this case the additional groups may have been compensated for by a simultaneous disappearance of histidines normally titrating in the same range. The titration range of iodohistidine is not known.

The sedimentation constant of iodinated albumin was increased by the amount predicted from the increase in molecular weight and density (that is, 20 per cent for a preparation that contains 34 atoms of iodine per molecule).[3] Sometimes a faster component was observed. This appeared to be an aggregate not necessarily related to the iodination process, but related rather to improper treatment of the protein either before or after iodination. Since such aggregates have very different biological behaviors and disappear rapidly from the circulation, ultracentrifugal evidence of homogeneity would appear to be very desirable for any labeled protein.

The determination of the biological activity of a protein after iodination is, of course, fundamental for its successful use as a tracer of the natural

protein. The results obviously depend on the essentiality of the groups reacted, and they cannot be generalized, but must be determined for each protein and, in fact, for each function of the particular protein. Thus, iodinated serum albumin has lost its sulfhydryl group. This should not affect its osmotic properties. In addition, it does not seem to affect its rate of catabolism markedly. Obviously, this loss will inhibit any function, such as heavy-metal detoxification, which is dependent upon the SH group, unless this group can be restored *in vivo*.

Frequently, loss of activity occurs progressively, as in the case of pepsin (FIGURE 4). In these cases it is difficult to determine the exact relation between the biological activity and the label, since one is probably measuring two averages that are grouped over different portions of the molecular population. In the experiment cited (FIGURE 4), many mechanisms are

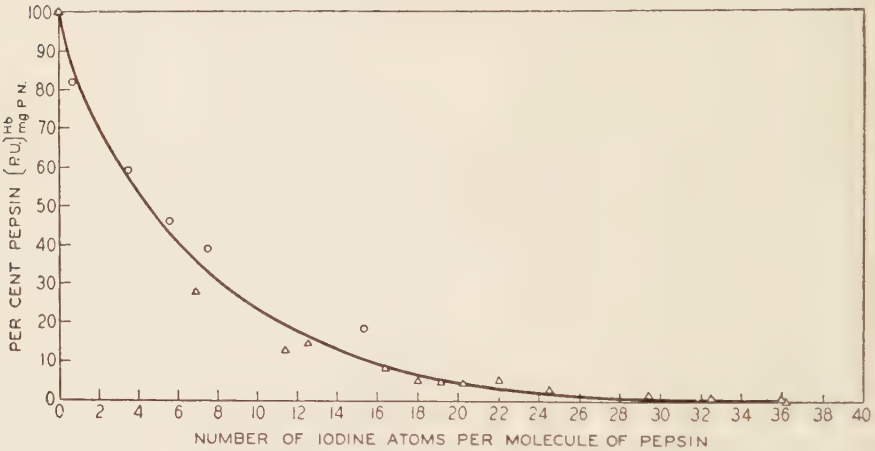


FIGURE 4. The effect of bound iodine on the specific activity of pepsin. The circles represent iodination in a solution buffered at *p*H 6 with acetate; the triangles indicate iodination in an unbuffered solution. Reproduced by permission from *The Journal of General Physiology*.[16]

compatible, but a few are excluded. Thus, it is obvious that inactivation cannot result only from substitution in an essential site if this site has equal reactivity toward iodine as the other sites, because simple blocking of this site should reduce enzymatic activity to zero *linearly* with iodine content when all the 16 tyrosines are covered (32 atoms of iodine), whereas a logarithmic relation is actually observed. However, by assuming one site to be essential for activity, and also assuming that in the other sites substitution merely *depresses* the activity, the observed curve could be formulated, presupposing equal iodine reactivity for all sites. (Of course, the *circles* in FIGURE 5 do not fit this formulation, since they appear to be extrapolating to measurable activity at an iodine content stoichiometric for tyrosine.)

An alternative simple explanation would assume sites of varying reactivity, the enzymatic site being among the most reactive. Thus, activity

would decrease more rapidly in the beginning; then, as the percentage of more reactive sites decreased, the rate of inactivation would decrease so that activity finally disappeared completely only upon saturation of all sites. A model for this mechanism exists in the greater reactivity of the second ortho substitution in each tyrosine. Thus, as iodination proceeds, competition by these more reactive sites increases. However, this effect alone could not explain the observed curve, since products with 8 iodine atoms could not have substituted more than half of the 16 tyrosyl groups, and necessarily would have more than 50 per cent reactivity, whereas only 30 per cent activity is found.

The above arguments serve merely to show the complexity of the problem and the lack of sufficient data for its complete understanding. For most tagging purposes, however, the important questions are: (1) Can a labeled active pepsin molecule be prepared? (2) How active will the labeled molecule be? FIGURE 4 indicates definitely that lightly labeled pepsin is active. Now, assuming our earlier suggestion of 2 atoms per molecule as a practical labeling level, and further assuming equal reactivity of all sites, statistically, one seventh of the molecules should be unlabeled.* However, using Herriott's estimate that one third of the iodine exists as diiodotyrosine,[29] we might then estimate that about one half of the molecules are unlabeled. Since at this level of labeling the average activity is 70 per cent of the original, then the labeled half of the molecules must contain, on an average, 70 minus 50, or 20 per cent of the original activity. Of course, some of the labeled molecules are completely inactive (perhaps 2 out of 16, or one quarter of those labeled), so that then three quarters of the label is on three eighths of the molecules containing (still on the average) 50 per cent of the specific activity of the starting material.

Some Practical Considerations and Notes of Caution in Methodology

I^{131}, as received from Oak Ridge, Tenn., is carrier-free, but it contains small amounts of sulfite to maintain it in the reduced state. Since sulfite oxidizes slowly in air, old lots may contain no sulfite and, consequently, some oxidized iodine, probably as iodate. Iodate is not in ready equilibrium with iodide and, consequently, may not be available for iodination. Therefore it is advisable to add a drop of dilute acid before adding carrier iodide or KI_3. On the other hand, an excess of sulfite in the solution of I^{131} will consume active iodine and thus alter the expected stoichiometry of the reaction. Whether an appreciable amount of sulfite exists can be noted readily during the addition of KI_3; that is, does the radioiodine solution decolorize the first increment of KI_3 added to it?

Various methods of iodination have been developed; all of them utilize iodine in the +1 oxidation state as the active species. The active iodine may be preformed as in KI_3 reagent,[3, 35, 36] or may be prepared by oxidation of iodide with nitrous acid,[37] iodate,[23] or hydrogen peroxide[38] just before use. As indicated above, KI_3 appears preferable, in spite of its smaller yield,

* Oncley has discussed the statistical distribution of label over identical reactive sites.[34]

because it possesses fewer side effects, and its activity can be buffered in each individual case by controlling the *p*H and the concentration of iodide. This makes it possible to follow a general precept in protein chemistry of always using the mildest conditions possible. On this basis it also would seem desirable to iodinate in the cold, although data to substantiate this are lacking.

Iodination is usually performed in the *p*H range of 8 to 10, which should provide for a variation of 2 orders of magnitude in the rate of iodination. It would seem desirable to choose a *p*H at which iodination proceeds at a rate readily followed visually. Too-rapid iodination may result in the reaction's taking place more rapidly than mixing can occur, thus labeling the molecules unevenly.

The stoichiometry of iodination should be determined for each set of conditions, since this permits an estimate of oxidative side reactions; that is, the amount of active iodine consumed, as measured by thiosulfate titration, should be compared with the iodine content of the protein (TABLE 4).

TABLE 4

IODINATION OF HUMAN SERUM ALBUMIN WITH SMALL AMOUNTS OF IODINE

	3 equiv./mol	6 equiv./mol
Albumin, gm.	4.48	4.75
Dissolved in water, ml.	20	20
M Na_2CO_3 added, ml.	15	15
M $NaHCO_3$ added, ml.	5	5
*p*H of solution	10.16	10.16
0.1 *N* I_2, 0.2 *M* KI added, ml.	2	4.29
Stoichiometry (equiv./mol)		
Iodine content found	0.77	2.25
Iodine added	3	6
Consumed for oxidation	1.46	1.50

The introduction of 1 gm. atom of iodine requires 2 equivalents of iodine; that is, one I^+. Any excess iodine consumed must have been used in oxidative side reactions. Usually an average of 1 or 2 atoms of iodine introduced per molecule of protein provides assurance that the label exists in fact on the protein species being investigated, while minimizing alteration due to the label. However, as further proof that such alteration has not occurred, it would seem desirable to show that a severalfold variation in degree of labeling does not affect the biological results obtained.

The iodination reaction may be stopped at any desired point by the addition of sulfite. In any case a small amount of sulfite, not to exceed the original iodine titer, should be added to reduce labile iodoprotein complexes before removal of the free iodide by dialysis or by ion exchange. Since some proteins, such as albumin, bind iodide appreciably, dialysis against a competing salt is more effective than by distilled water. Carrier iodide also may be added to equilibrate with labile iodine, and it then may be removed by

dialysis or by precipitation of the protein. The percentage of unbound iodine usually may be determined by precipitating the protein with trichloroacetic acid (TCA). Since most proteins show a slight solubility in TCA, small amounts of radioactivity remaining in the trichloroacetic supernatant may not be free iodide. This can be ascertained by shaking the supernatant with free iodine in an organic solvent and noting whether the activity is transferred to the organic layer.

Whenever possible, criteria of effectiveness of the iodination procedure should include an appropriate biochemical or physiological test. When these do not exist, some physicochemical criteria such as ultracentrifugal homogeneity are useful. Studies of the rate of disappearance *in vivo* that show a single slope in a semilogarithmic plot are particularly useful since, frequently, the organism rapidly catabolizes altered protein. This may be most sensitively detected as a burst of iodide released in the first hours following injection.[37]

In summary, the above discussion of the iodination of proteins has emphasized the principles involved. No detailed methodology has been given, since I believe that the preparation of any new protein derivative must be considered a research project in itself, one that would require experimentation in preparative procedures and careful characterization of the products, before reliance could be placed in physiological applications.

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THE BEHAVIOR OF I^{131}-LABELED PLASMA PROTEINS *IN VIVO*

By A. S. McFarlane

The National Institute for Medical Research, London, England

The technique used for labeling plasma proteins with radioiodine (I^{131}) used at the National Institute for Medical Research has not been changed in any essential in several hundreds of iodinations over the past few years. This method of iodination[1] may be described briefly as follows:

Free labeled iodine is first prepared in true solution in an amount equivalent to a final binding of a single statistical mean atom of iodine for every molecule of protein to be labeled. Just before mixing with the protein solution the latter is buffered with glycine to *p*H 9.3. The mixing is done by injecting the free iodine solution at high velocity into a counterstream of buffered protein in order to reduce uneven distribution of the label. Carrier iodide is added, and the *p*H quickly reverts to between 7 and 7.5 as the mixture is passed through an ion-exchange column to remove this iodide along with unbound I^{131}. An excess of unlabeled protein or, more commonly, of whole serum protein is added to the effluent to act as carrier and to reduce damage by self-radiation. The procedure is rapid and convenient. Solutions for clinical use are finally Seitz filtered.

Comparison with C^{14}-Labeled Proteins

Experiments have already been described in which the *in vivo* behavior of albumins and γ-globulins labeled in this way have been compared with similar C^{14}-labeled proteins in rabbits[2] and rats.[3] Almost identical behavior was observed in both species over periods of three to four weeks.

Another kind of comparison has recently been carried out by Humphrey and the author with the results shown in FIGURE 1. In this case, C^{14} labeled pneumococcus Type III antibody globulin, of which 70 per cent was specifically precipitable with capsular polysaccharide, was isolated from the serum of a hyperimmune rabbit which had previously received C^{14}-amino acids.[4] This was mixed with globulin similarly prepared from another immunized rabbit and labeled with I^{131}; 40 mg. of labeled normal and immune γ globulins were injected into a normal rabbit. The low and rapidly declining concentrations of antibody in the plasma of this animal could not be measured readily by conventional means; however, by the preliminary addition of 4 mg. of inactive carrier antibody to each 0.4 ml. sample of serum, and specific precipitation with pneumococcus polysaccharide, we obtained precipitates that contained diminishing quantities of radioactive antibody. These could be handled quantitatively and their radioactivities measured separately; the I^{131} γ radiation by direct measurement on the dry precipitate and the C^{14} β radiation after combustion to CO_2. Values for the ratio of these two activities have a high accuracy since, as shown at the bottom of FIGURE 1, this ratio is independent of errors in the times of sampling or of the recovery

of precipitates. Clearly, the rabbit did not discriminate between the two labeled antibodies.

In a second test, aliquots of the same mixture of labeled globulins were injected into 6 rats. After 10 days, immunity developed to the heterologous protein, and antibody globulin began to disappear precipitously from the plasma. After 13 days, when the plasma concentration had fallen to 2.0 μg./

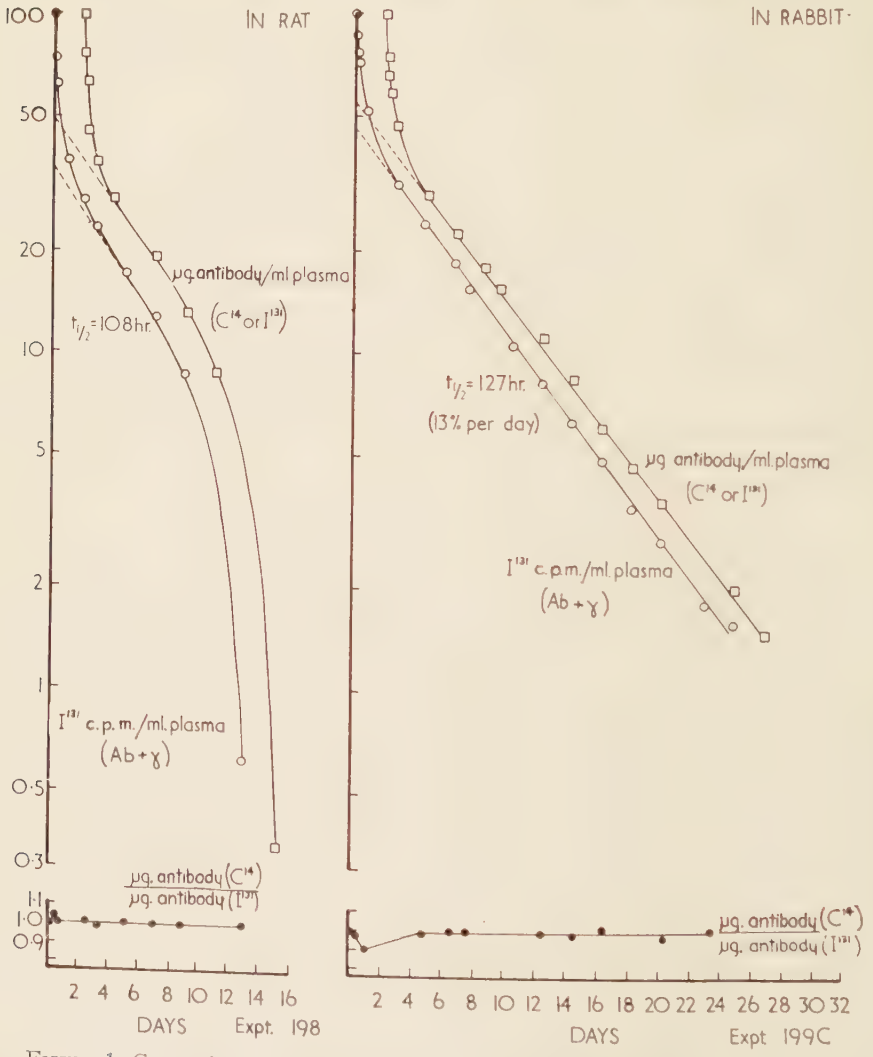

FIGURE 1. Comparison of mixed I^{131}- and C^{14}-labeled rabbit antibodies *in vivo*. Concentrations of antibody and I^{131} c.p.m./ml. in the plasma of recipient animals plotted against time after the injection of aliquots of a mixture of C^{14}- and I^{131}-labeled antibody to pneumococcus Type III. Values are given as percentages of initial values and, in the case of the concentrations of antibody, should be multiplied by 4 to give μg./ml. Note the arbitrary displacement of the two curves to avoid overlapping.[8]

ml., or 0.3 per cent of the starting value, enough radioactivity of both kinds was still measurable in the specific precipitate to show that the ratio remained unchanged. Thus, in the operation of the immunity process, whatever its essential nature may be, it is evident that no discrimination had taken place between molecules labeled by the biosynthetic and *in vitro* procedures used here.

The view is now widely held that most, if not all, biological processes are not significantly affected by substituting C^{14} labeled substances for their natural carbon counterparts. On the other hand, major effects on the physical and chemical properties of tyrosine may be expected to follow ring substitution of I^{131}, and the fact that *in vivo* tests failed to show discrimination against a globulin labeled in this way suggests that the mechanisms for recognizing altered homologous proteins in the blood or interstitial fluids must be relatively insensitive. They must certainly be much less insensitive than those that operate at the amino acid level, since plasma-free iodotyrosine is unacceptable for the synthesis of proteins.

Urinary Excretion of I^{131}

As protein metabolism proceeds in an animal that has been injected with a I^{131}-labeled plasma protein and whose thyroid has been saturated with inactive iodide, the label appears quantitatively in the urine in diffusible form. Inability to reutilize the label in the formation of plasma protein has been demonstrated by feeding excessive amounts of I^{131}-labeled plasma proteins and by subsequent failure to find any significant concentration of labeled proteins in the plasma.[2]

Activity appearing daily in the urine in metabolic experiments with I^{131}-labeled proteins has most frequently been expressed as a fraction of total activity retained in the animal, a total which, in effect, is the sum of protein-bound activities in plasma and interstitial fluids. The proportion of activity in the urine expressed in this way has been observed to fall steadily in the first few days during which time massive net transfer of labeled molecules is taking place from the plasma into the extravascular fluid (FIGURE 2). This fall is believed by some to indicate that denatured labeled molecules were present in the injected material and are being rapidly broken down; this, indeed, is known to be the fate of molecules deliberately altered.[5] However, using an isolated perfused rat liver preparation, Gordon has shown that I^{131}-labeled γ globulin that was mildly heat-denatured and remained soluble nevertheless was removed rapidly from the circulating plasma and that its label was liberated in diffusible form.[6] On the other hand, untreated I^{131}-labeled albumin preparations that uniformly have only quite negligible proportions of their activity removable in this way by the perfused liver all show the same pronounced fall in the daily fraction excreted in the first few days.

When urinary activities were expressed as fractions of plasma activities alone, constant values were obtained throughout the experiments in which labeled albumins, but not γ globulins, were used in rats,[3] rabbits,[7] and humans[8] (FIGURE 2). This constant relationship is taken to mean that meta-

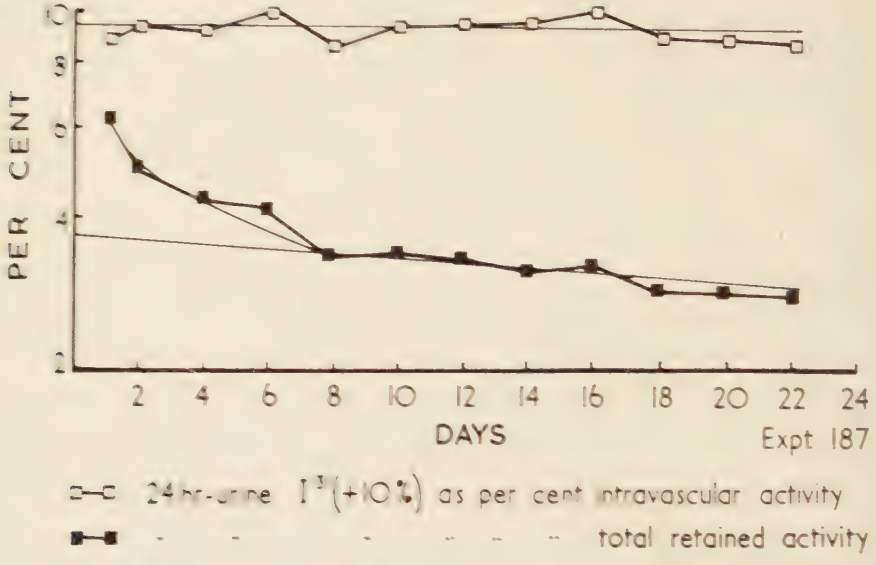

FIGURE 2. The excretion of I^{131} by human [illegible] labeled human albumin. The urine activities are those of young adult males in normal occupations. The fecal excretions were assumed to be 10 per cent of the urinary values.[5]

bolic breakdown of albumin occurs in the plasma or in close proximity to it. Iversen and Yalow[9] arrived at the same conclusion for ascitic patients by studying urinary excretion after injecting I^{131} into the peritoneal cavity. The constant percentage of plasma activity that is excreted daily in urine and feces we propose to call the *katabolic rate*. The approximate metabolic rate for homologous albumin in the human is 10 per cent, in the rabbit 20 per cent, and in the rat 60 per cent per day, with variations of ± 20 per cent between individuals of the same age and weight. In absolute terms, these rates correspond to 12, 0.8, and 0.25 gm. albumin ± 20 per cent, respectively, broken down per day in adults of average weight. The metabolic rate is generally higher in immature animals than after maturity is reached, and it appears to be little affected, if at all, by the level of protein nitrogen in the diet.

Apparent Rates of Replacement

It has been assumed that similar absolute estimates of the metabolism of albumin in normal animals can be derived from half lives of plasma activity. Sterling[10] and others have indicated that the slope of the exponential part of the plasma-specific activity-time curve corresponds to the breakdown of a constant fraction of lymph plus plasma protein, and that extrapolation of this part of the curve to cut the activity ordinate at zero time enables the mass of extravascular protein to be measured in terms of total plasma protein. Analyses of the lymph-plasma situation in the animal made by

several workers,[9, 11, 14] however, shows that this is not permissible for the following reason:

As injected labeled molecules first enter the various interstitial fluids, which may be visualized as constituting a single equivalent lymph compartment, the mean protein-specific activity increases, while its value in the plasma falls. Ultimately, the two values become equal, and net transfer of labeled molecules across the boundary of capillaries is discontinued. This equality can persist only if specific activities in both compartments decline at the same rate; that is, in the unlikely event that metabolic breakdown occurs in both compartments in proportion to the mass of protein in each. If breakdown occurs mainly or exclusively in the plasma, specific activities at all times will be lower in this compartment, and a persistent net transfer of labeled molecules must occur from the lymph back into the plasma. This transfer will have the effect of prolonging the half life of plasma activity beyond a value corresponding to true metabolic breakdown.

Accordingly, the slope of the observed plasma-activity curve represents only an apparent replacement or breakdown rate, and determination of the true value requires a knowledge of the rate of redistribution of labeled molecules between the two compartments. This information can be obtained from measurements of plasma and urinary activity in the first few days after injection[3] or from graphic analysis of the plasma activity curve alone throughout its whole course,[14, 15] both derivations being independent of whether the mechanism of return of labeled molecules to the plasma is by fluid flow via lymphatic channels or by reverse diffusion through the capillaries. However, both of these processes depend on an assumption of rapid mixing in the equivalent lymph compartment,[16] which has not been demonstrated.

Metabolic rates of the globulins. I^{131}-labeled rat, rabbit, and human γ-globulins contrast with albumins of the same species in showing steadily declining metabolic rates with time. Thus, in the case of rat γ-globulin in the rat, values start at 18 to 20 per cent and fall to 12 to 15 per cent at 15 days.[3] This kind of behavior would be expected if a mixed population of labeled molecules had been injected, the initial metabolic rate representing the mean of rates characteristic of all labeled molecules present, and the later values representing principally the rates of molecules that survive longest. Alternatively, such a situation might arise if a proportion of the injected labeled molecules were metabolized in the lymph compartment. The former view is favored by the result of an experiment with "screened" rat γ-globulin; that is, labeled γ-globulin molecules that had survived for 10 days in one rat and were then transferred to another. The initial metabolic rate in the second animal corresponded to the 10th day rate in the first animal and, subsequently, the rate of fall was much reduced.[17]

However, the view that γ-globulins so far examined are all heterogeneous in a metabolic sense must be qualified by one unexplained fact. Campbell, Cuthbertson, and Matthews[3] have shown that the apparent replacement curve of a mixture of labeled proteins (rat albumin and γ-globulin) cannot be expressed as a single exponential function in any part of its course, at

least not until the more rapidly metabolized component, albumin, represents a negligible proportion of the labeled molecules remaining in the plasma. On the other hand, I^{131}-labeled γ-globulins of rats, rabbits, and humans, when examined alone, all showed long single exponential replacement curves. Further investigation is clearly required.

We have also observed that labeled γ-globulins, as a class, take longer to achieve apparent equilibrium between plasma and lymph than do similarly labeled albumins. Thus, the initial curved portions of plasma activity curves in the case of rat and human γ-globulins merge with the exponential portions after the sixth day, whereas albumin curves in both species often merge at the third day. This also could be due to greater metabolic inhomogeneity of the γ-globulins.

Different preparations of γ globulin, all iodinated by the same technique as far as possible, have frequently shown different degrees of homogeneity by one or both of the above criteria, which suggests that extreme care is necessary in the isolation of proteins for iodination. Our practice is to verify, by paper electrophoresis and subsequent scanning of the paper, that the radioactivity migrates with a single electrophoretic component of the carrier serum that is added to our I^{131}-labeled protein preparations. However, electrophoretic purity does not necessarily imply metabolic homogeneity in the sense discussed here and, of course, the shortcomings of paper electrophoresis as a technique for recognizing the presence of protein contaminants should be kept in mind.[16]

Discussion and Summary

It is evident from comparisons of C^{14}- and I^{131}-labeled albumins and γ-globulins, especially when performed in the same animals, that the iodine label can be useful for many biological investigations that involve intact protein molecules. The advantage that this label offers is not solely due to the circumstance that alternative methods of isotopic labeling are, for the most part, biosynthetic and are therefore inefficient in the case of plasma proteins or precluded in the case of human proteins on ethical grounds. The label has the additional and unique advantage that it is promptly and quantitatively excreted from the animal following the breakdown of the injected protein.

We discussed evidence that indicates that the site of metabolic breakdown of albumins and, possibly, of all plasma proteins, *if it comprises any significant pool of the particular protein concerned*, cannot be other than in the plasma itself, or at least cannot be separated from it by any significant permeability barrier. If this is true, the rate of excretion of the label can be regarded as a direct and absolute measure of the amount of the particular protein that is metabolized daily, and average figures are given for this in the case of rat, rabbit, and human albumins. Reference is made to alternative methods of determining absolute metabolic rates also.

In using the label, the presence of denatured molecules that may arise, for example, by iodinating proteins that have been heated to 55° C., or that have been in contact with cold methanol[1] should be avoided. In addition,

the chemical purity of the proteins to be labeled is essential if currently intelligible results are to be obtained over the full period of a metabolic experiment.

Evidence is presented that suggests that, although labeled γ globulins of several species examined were homogeneous by paper electrophoresis, they comprised a population of molecules that was metabolized at different fractional rates. To what extent such heterogeneity may be intrinsic or the result of iodination is not known. Self-radiation to a level of 50,000 rads did not increase it. By the same metabolic criteria, labeled albumins appeared to be homogeneous.

The author is indebted to several collaborators for permission to present some hitherto unpublished data in this communication.

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COMPARATIVE METABOLIC FATE OF CHEMICALLY (I^{131}) AND BIOSYNTHETICALLY (C^{14}- OR S^{35}-) LABELED PROTEINS*

By Patrick D. Goldsworthy and Wade Volwiler
Department of Medicine, University of Washington, Seattle, Wash.

Introduction

In order to establish whether or not any differences exist among the metabolisms of proteins labeled in different ways, satisfactory criteria of measurement must be chosen. We believe that the determination of the apparent life spans of protein molecules has proved to be the best criterion studied thus far for the plasma proteins of mammalian species. However, an understanding of the inherent theoretical and experimental errors[1] is necessary for the interpretation of measurements of the life span or turnover of protein molecules.

These measurements have been made for plasma proteins by both (1) the Hevesy labeling procedure[2] and (2) the endogenous labeling procedure.[2] The Hevesy procedure consists of the administration of a labeling isotope that already has been chemically attached to or biosynthetically incorporated into the protein itself. The endogenous procedure consists of administration of such an isotope that has been synthesized into the structure of one or more of the protein's constituent amino acids. The Hevesy procedure may employ either the *in vivo* intrinsic labeling technique for introducing C^{14} or S^{35} into the protein or the *in vitro* extrinsic labeling technique for chemically attaching I^{131} to the protein. The endogenous procedure is, by definition, an *in vivo* intrinsic labeling technique that may be used to introduce C^{14} or S^{35} into the protein.

Methodology of C^{14} and S^{35} Biosynthetic (Intrinsic) Labeling Technique

Hevesy Procedure of Administering in Vivo-Labeled Protein

Step 1: administration of labeled amino acid to donor. One or more isotopically labeled amino acids is given to a donor animal, which results in the biosynthetic labeling of the plasma proteins to be transfused to a recipient animal. The two labeling amino acids selected by our laboratory have been those which would exchange their labeled positions with other amino acids only slightly—if at all. S^{35}-labeled cystine was chosen because the sulfur is not transferred to methionine[3-5] and lysine, because only very small quantities of its ε-carbon are contributed to other amino acids.[6-9] Through

* All of the published and unpublished data from our laboratory referred to in this paper were obtained through the support of grants from the National Institute of Arthritis and Metabolic Diseases, Public Health Service, Bethesda, Md., from A. H. Robins Co., Inc., Richmond, Va., and through funds for Research in Biology and Medicine accruing through Initiative 171 of the State of Washington.

the use of these amino acids and their labeled positions, we were able to achieve an uptake and loss of isotope by the protein almost exclusively in the form of the administered amino acid. There was also less of a catabolic loss of activity from the structure of these molecules than would have been the case with most other amino acids.

The dose of administered radioactivity should be as high as practicable in order to compensate for the dilution and loss of isotope that occurs in the donor animal. Dilution of the isotope may be reduced by selecting a small donor animal. Our laboratory has found administration of 1-cystine-S^{35} in doses of 20 μc./kg. for humans and 150 μc./kg. for dogs, and 1-lysine-6-C^{14} in doses of 160 μc./kg. for dogs to be adequate.[3, 10]

The amino acid is administered as a single dose by either the intravenous or oral route. The oral route has found extensive application due to its simplicity and to the high efficiency of the intestinal absorption of amino acids.

Step 2: administration of labeled donor plasma proteins to the recipient. When, following administration of the labeled amino acid, the concentration of the isotope has reached a maximum in the specific protein to be studied, blood is withdrawn from the donor. The quantity taken should be sufficient to permit the administration to the recipient of an adequate level of radioactivity within the protein being measured, whether this labeled protein is given isolated from, or mixed with, the other plasma proteins. In experiments where only the half life or turnover time of the protein is to be studied, the *mixture* of labeled plasma proteins may be administered intravenously to the recipient. However, if the pool size and replacement rate are to be measured also, it is essential to administer only the *single* protein molecular species to be studied. If a sufficiently small donor animal is employed, all the plasma obtained by exsanguination may be used. Our laboratory has administered cystine-S^{35}-labeled plasma proteins in doses of 0.05 to 0.3 μc./kg. to humans and 1 to 3 μc./kg. to dogs, and lysine-6-C^{14}-labeled plasma proteins in doses of 0.5 to 1.7 μc./kg. to dogs.[3, 10]

Step 3: isolation of recipient's plasma proteins. Periodic samples of blood are withdrawn from the recipient in sufficient quantities to permit the isolation of each protein being studied from the mixture of plasma proteins. In this resolution of protein species and their associated specific activities we have usually employed fractionation procedures[3] where some proteins may be isolated in sizeable quantities (1.5 gm. of albumin from 50 ml. of plasma) and others in minimal quantities (50 mg. β_1 lipoprotein from 50 ml. of plasma). However, it should be possible to employ chromatographic and zone-electrophoretic procedures to calculate graphically the specific activities of proteins resolved from small samples of plasma (2 to 5 ml.). The use of a single isolation technique usually yields a less homogeneous protein product than does a successive combination of several techniques, each utilizing one of the following four different physical or chemical properties of the protein for its separation: (1) differential solubilities of the proteins or their complexes or derivatives in salting out techniques,[11] or in techniques of controlled *p*H and changes in ionic strength at room temperature[12, 13] or

at reduced temperatures in a solvent system;[13-17] (2) molecular weights and densities of the proteins in ultracentrifugal sedimentation and flotation techniques;[18] (3) net charges of the proteins in free[19, 20] or zone[21-23] electrophoretic resolution; and (4) chemical reactivity of the proteins in chromatographic resolution.[24, 25]

The additional purification possible when a protein obtained by differential solubility is subjected to starch zone electrophoresis may be illustrated by the resolution of Cohn Fraction V[16] obtained in our laboratory (FIGURES 1 and 2).

Following the first blood sampling, which should occur immediately after injection, subsequent samples should be withdrawn at 1- or 2-day intervals during the first week in order to delineate adequately the nonexponential portion of the isotope turnover curve. In the dog and human, the apparent

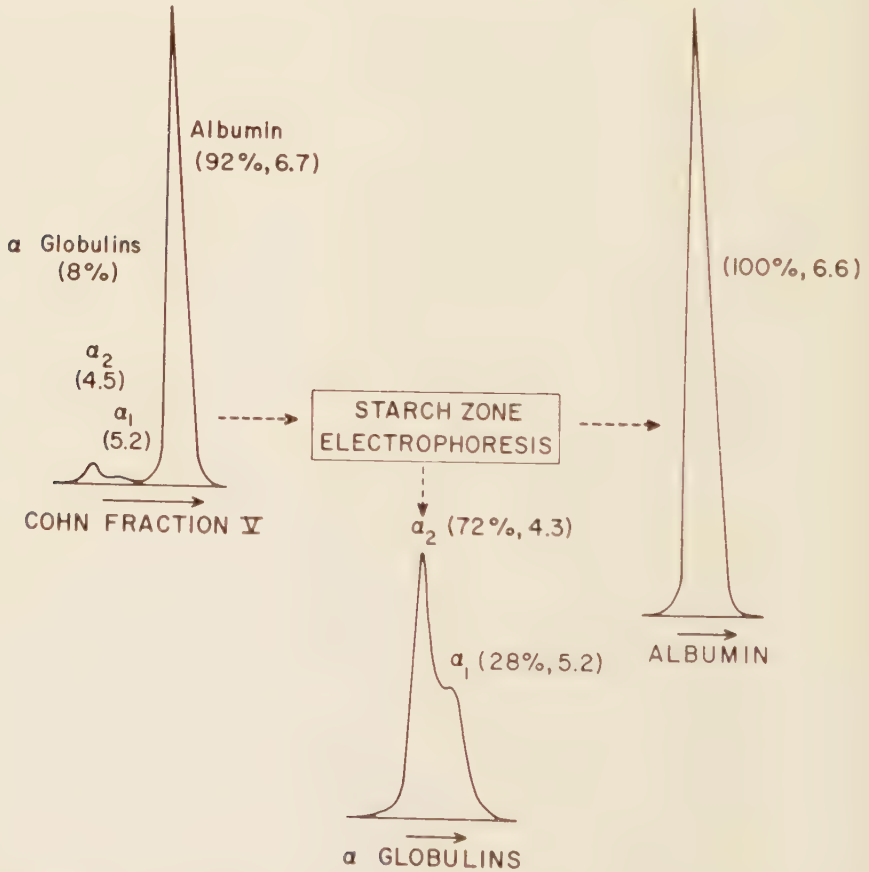

FIGURE 1. Purification of α-globulins from albumin as obtained by resolving an inhomogeneous Cohn Fraction V by starch zone electrophoresis. Mobilities and compositions determined by free electrophoresis are shown in parentheses for Fraction V before purification and the 2 fractions isolated by zone electrophoresis.

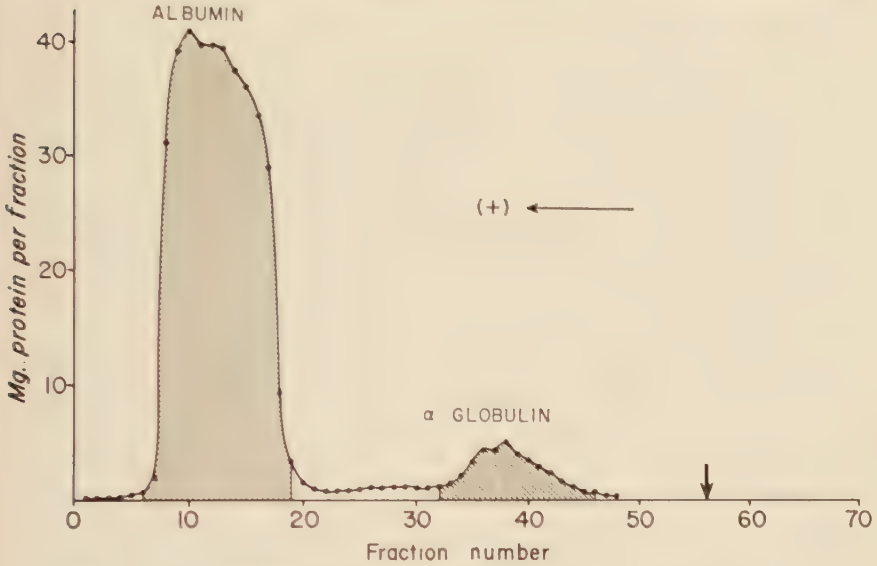

FIGURE 2. Zone electrophoretic purification of proteins in Precipitate V from Cohn fractionation method 6 (dog No. 4). Veronal citrate buffer, pH 8.6, specific conductance 0.00253 mhos (0.5 M diethylbarbituric acid, 0.00765 M sodium citrate, adjusted to pH with sodium hydroxide and to specific conductance with sodium chloride). The supporting medium is 175 gm. of potato starch granules as a block 38 cm. long, 39.5 mm. wide, and 14.5 mm. thick. The electric field has a difference of potential of 112 volts at the electrodes with a current of 27 m.Amp. per block. Current applied for 36 hours; amount of protein applied at origin (heavy arrow) is 2 to 2½ ml. of a solution containing 800 mg.; amount of protein recorded, 600 mg. The direction of the current is indicated by the thin arrow. The block was cut into 5 mm. segments from each of which the protein was eluted with 0.1 M of sodium chloride. Aliquots were taken for protein analysis by the Folin-Ciocalteu method.[3] The hatched areas show which eluate fractions were pooled for isotope analysis of each protein component isolated.

metabolic degradation or rate of turnover may be established by sampling blood at 2- to 5-day intervals after completion of the rapid mixing phase and before the isotope level becomes sufficiently low for isotope reincorporation to be significant (FIGURE 3).

Step 4: isotopic assay of isolated proteins. Oxidation of different aliquots of the protein to carbon dioxide and sulfate[3] permits separate isotopic assays of $BaCO_3$ and benzidine sulfate, respectively, with resulting increases in the specific activity of the fraction over that of the whole protein. This increase also permits the separation of C^{14} from S^{35} in doubly labeled proteins, and it thus allows these isotopes to be measured independently. The assay also may be made on thin unoxidized protein films filtered from finely homogenized suspensions of lyophilized protein in ether. If the protein is labeled with two isotopes having significantly different isotopic decay rates, the contribution of each to the observed combined radioactivities can be obtained without separating the isotopes. Several measurements of the combined radioactivi

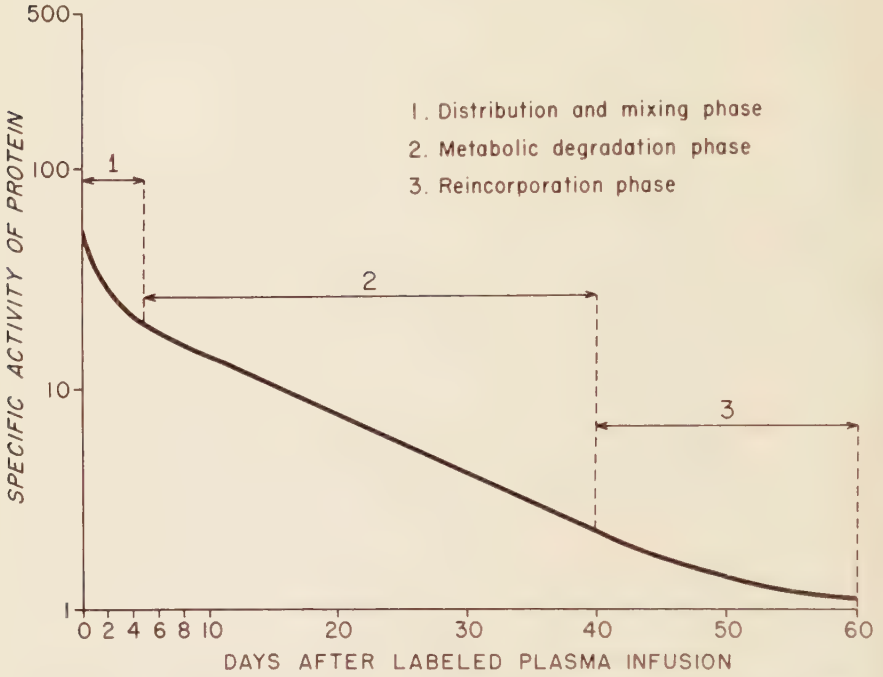

FIGURE 3. Schematic turnover curve showing the rate of loss of isotope from a homogeneous human plasma protein following the intravenous administration of labeled protein.

ties at different times are made, from which, by simultaneous equations, it is possible to calculate the radioactivity due to each isotope.

Step 5: graphical calculation of half life, turnover time, pool size, and replacement rate. The observed specific activity data, appropriately related to the weights of protein, carbon, or sulfur, are plotted semilogarithmically as ordinate values against time as the abscissa. Such an isotope die-away curve may normally be divided into the 3 phases shown in FIGURE 3. The first of these is a rapid distribution or mixing phase during which the labeled protein is assumed to approach equilibrium (1) immediately after administration, with the intravascular plasma protein,[26-28] (2) then, with the rapidly equilibrating lymph protein,[27-30] (3) next, with the less rapidly circulating lymph protein of the interstitial spaces,[26, 27, 29-31] and (4) finally, perhaps, even with the proteins within the intracellular spaces.[29] The duration of the first phase may be considerably extended in the case of mammals having a large volume of edema fluid that represents an increased extravascular space.[2, 29, 32-34] Abdou and Tarver[29] have concluded that plasma protein reaches equilibrium with the tissue or tissues that cause its breakdown after a period of 2 or 3 hours in the normal rat. This was indicated by the appearance of maximum radioactivity in the expired CO_2 2 to 3 hours following the intravenous administration of labeled plasma protein.

The second phase is the straight-line, exponential portion of the curve presumed to reflect primarily the rate of metabolic degradation of the labeled protein.[3, 26, 28, 29] The third phase, which is demonstrable only if the experiment continues for a sufficient time, is assumed to reflect the re-entry of the isotope into the protein. If any of these phases includes more than 1 rate process, the plotted data will follow a curved line such as the top curve in FIGURE 4.

Since the sum of several exponential processes cannot itself be an exponential function, one is justified in attempting to resolve the plotted curve graphically into its exponential components[32, 35-37] as shown in FIGURE 4. This is accomplished by subtracting the extrapolated values

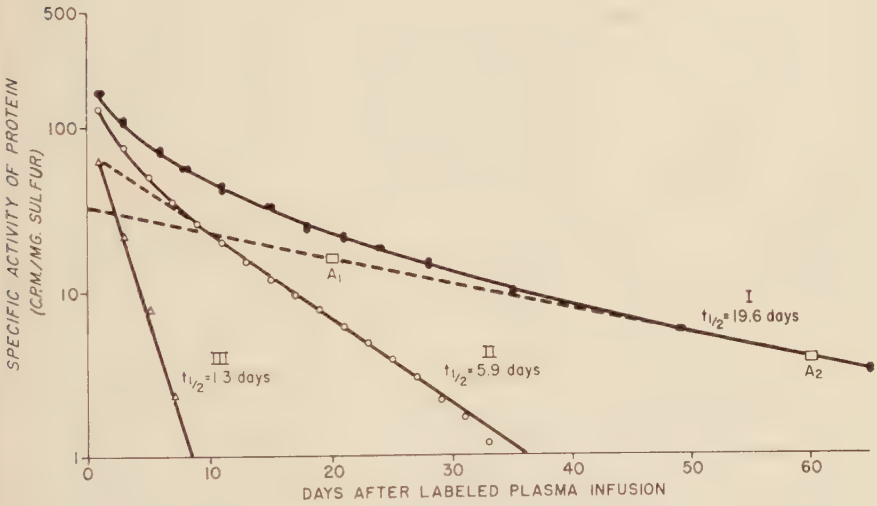

FIGURE 4. Graphic analysis of a nonexponential turnover curve of S^{35}-labeled γ-globulin (dog).

The half life of component I is calculated from $A_1 = 16.4$ c.p.m./mg. S (on day 20) and $A_2 = 4.0$ c.p.m./mg. S (on day 60):

$$t_{1/2} = \frac{(\log 2)(60 \text{ days} - 20 \text{ days})}{\log 16.4 \text{ c.p.m.} - \log 4.0 \text{ c.p.m.}} = 19.6 \text{ days}$$

(broken line) of the specific activity for the slowest rate process (I) of the top curve from the actual values (curved portion of top curve) thereby yielding Curve II, which represents an intermediate rate process. Repetition of this subtraction for Curve II yields Curve III, which reflects the fastest rate process. The resulting 2 derived curves indicate that the nonmetabolic disappearance of the plasma protein label from the circulating plasma is due to a minimum of 2 processes (II and III) that probably reflect rates of plasma protein equilibration with 2 different "body compartments." Use of such graphical data to calculate half lives, turnover times, pool sizes, and replacement rates is summarized in the sample calculations of TABLE 1, which are taken from the data of Margen and Tarver[38] and of Sterling.[39]

TABLE 1

EXAMPLE CALCULATIONS OF VALUES RELATED TO PROTEIN TURNOVER OF I^{131}-LABELED ALBUMIN

QUANTITIES MEASURED	Margen and Tarver[28]		Sterling[29]	
	Administer 1 protein, count whole plasma	Administer whole plasma, count 1 protein (hypothetical calculation)	Administer 1 protein, count whole plasma	Administer whole plasma, count 1 protein (hypothetical calculation)
Dose of radioactivity injected	8.02×10^6 c.p.m.	———	6.75×10^6 c.p.m.	———
Plasma volume if determined by T-1824	———	2587 ml.	———	2446 ml.
Plasma radioactivity at zero time	3.10×10^3 c.p.m./ml. plasma		2.76×10^3 c.p.m./ml. plasma	
Intercept at zero time of the specific activity ordinate by the extrapolated exponential portion of the turnover curve	5.6×10^2 c.p.m./ml. plasma[a]		11.6×10^2 c.p.m./ml. plasma[b]	
Plasma concentration of specific protein	53 mg./ml. plasma		42 mg./ml. plasma	
QUANTITIES CALCULATED				
Half life of protein[c], $t_{1/2}$	15 days		11.2 days	
Distribution ratio of protein[d]	$\frac{8.02 \times 10^6 \text{ c.p.m. injection}}{5.6 \times 10^2 \text{ c.p.m./ml. plasma}} = 1.43 \times 10^4$		$\frac{6.75 \times 10^6 \text{ c.p.m./injection}}{11.6 \times 10^2 \text{ c.p.m./ml. plasma}} = 5.82 \times 10^3$	
S_{100}[e]	$\frac{5.8 \times 10^2 \text{ c.p.m./ml. plasma}}{3.10 \times 10^3 \text{ c.p.m./ml. plasma}} 100 = 18.71\%$		$\frac{9.0 \times 10^2 \text{ c.p.m./ml. plasma}}{2.76 \times 10^3 \text{ c.p.m./ml. plasma}} 100 = 32.61\%$	
Protein specific activity at zero time in plasma	$\frac{3.10 \times 10^3 \text{ c.p.m./ml. plasma}}{53 \text{ mg. protein/ml. plasma}} = 58.5$ c.p.m./mg. protein		$\frac{2.76 \times 10^3 \text{ c.p.m./ml. plasma}}{42 \text{ mg. protein/ml. plasma}} = 65.7$ c.p.m./mg. protein	

Quantity of rapidly circulating intravascular protein	$\frac{8.02 \times 10^6 \text{ c.p.m.} \times 10^{-3}}{58.5 \text{ c.p.m./mg. protein}}$ = 137 gm. protein	2587 ml. $\frac{53 \text{ mg. protein/ml. plasma}}{1000}$ = 137 gm. protein	$\frac{6.75 \times 10^6 \text{ c.p.m.} \times 10^{-3}}{65.7 \text{ c.p.m./mg. protein}}$ = 103 gm. protein	2446 ml. $\frac{42 \text{ mg. protein/ml. plasma}}{1000}$ = 103 gm. protein
Average protein specific activity at zero time in total body pool if mixing were instantaneously complete	$\frac{5.6 \times 10^3 \text{ c.p.m./ml. plasma}}{53 \text{ mg. protein/ml. plasma}} = 10.57$ c.p.m./mg. protein		$\frac{11.6 \times 10^3 \text{ c.p.m./ml. plasma}}{42 \text{ mg. protein/ml. plasma}} = 27.6$ c.p.m./mg. protein	
Size of total exchangeable protein pool	$\frac{8.02 \times 10^6 \text{ c.p.m.} \times 10^{-3}}{10.57 \text{ c.p.m./mg. protein}}$ = 759 gm. protein	$\frac{3.10 \times 10^3 \text{ c.p.m./ml. plasma}}{5.6 \times 10^3 \text{ c.p.m./ml. plasma}}$ 137 gm. protein = 758 gm. protein	$\frac{6.75 \times 10^6 \text{ c.p.m.} \times 10^{-3}}{27.6 \text{ c.p.m./mg. protein}}$ = 245 gm. protein	$\frac{2.76 \times 10^3 \text{ c.p.m./ml. plasma}}{11.6 \times 10^3 \text{ c.p.m./ml. plasma}}$ 103 gm. protein = 245 gm. protein
Turnover of protein pool	$\frac{100 \ln 2}{t_{1/2}} = \frac{69.3}{15 \text{ days}} = 4.62\%$/day		$\frac{69.3}{11.2 \text{ days}} = 6.19\%$/day	
Daily synthetic (replacement) rate	759 gm. protein $\times \frac{4.62\%/\text{day}}{100} = 35$ gm./day		245 gm. protein $\times \frac{6.19\%/\text{day}}{100} = 15$ gm./day	
Ratio of extravascular to intravascular protein	$\frac{759 \text{ gm. protein} - 137 \text{ gm. protein}}{137 \text{ gm. protein}} = 4.5^f$		$\frac{245 \text{ gm. protein} - 103 \text{ gm. protein}}{103 \text{ gm. protein}} = 1.4^g$	

[a] McFarlane[50] has pointed out that this value is erroneously low due to lymph albumin of higher specific activity re-entering the vascular circulation, thus raising the plasma protein specific activities and reducing both the slope of the exponential portion of the turnover curve and its extrapolated intercept at zero time.

[b] The erroneously low value due to effects in (*a*) is offset by an erroneously steep exponential turnover curve resulting from an experiment of too short duration.

[c] Half life ($t_{1/2}$) $= \frac{(\log 2) \cdot (n \text{ days between } sa_a \text{ and } sa_{a+n})}{(\log sa_a - \log sa_{a+n})}$

sa_a = specific activity of protein on day a.
sa_{a+n} = specific activity of protein n days after a.

[d] Represents the ratio of the total mass of the particular protein in intra- and extravascular compartments to the rapidly circulating mass of the same protein.[39, 40]

[e] Protein specific activity in the plasma at an arbitrary time (100 hr.) expressed as a percentage of the value at zero time.[39, 40] This reflects the early rate at which the labeled protein leaves the plasma compartment.

[f] Falsely high value resulting from errors discussed under (*a*).

[g] Reasonable value due to the compensating errors discussed under (*b*).

Hevesy Procedure of Administering in Vitro-Labeled Protein

The rates of turnover of proteins labeled chemically or extrinsically at reactive sites with isotopic reagents such as I^{131} are studied essentially in the same manner as are intrinsically labeled proteins. The major difference lies in the fact that, for extrinsic labeling, the protein to be administered is isolated in a nonradioactive form, which can then be labeled *in vitro* with an isotope concentration much higher than is feasible biosynthetically. Consequently, the extrinsic labeling technique is less wasteful of isotope. Since there is no reutilization of the isotope released from *in vivo* degradation of I^{131}-labeled proteins,[27, 32] which is not the case with S^{35}- or C^{14}-labeled proteins, the urinary isotopic excretion rate may be used to measure the rate of protein degradation,[27, 32] and it is not necessary to isolate the protein being studied, which is the only one that is labeled, from the other plasma proteins, none of which is labeled. In addition, the γ radiation from I^{131} is much more penetrating than the β radiation from S^{35} or C^{14}. Therefore it is possible, in the assay of protein-I^{131}, to avoid the involved preparations necessary for measuring protein-S^{35} and protein-C^{14}.

Endogenous Procedure of Administering Labeled Amino Acid

This biosynthetic labeling procedure requires that the protein turnover be studied in the same animal receiving the labeled amino acid. The same techniques as already discussed for the Hevesy procedure are used following

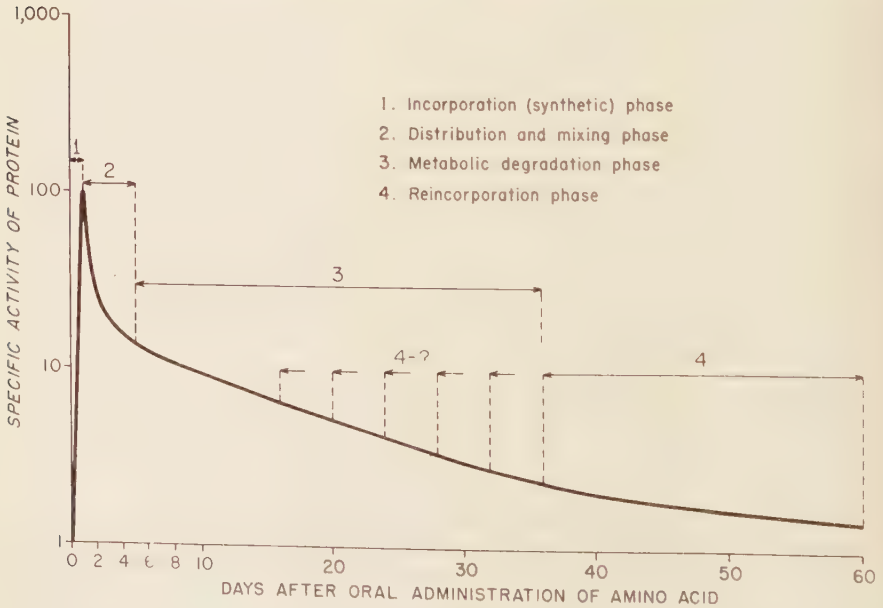

FIGURE 5. Schematic turnover curve showing the rate of change of isotope in a homogeneous human plasma protein following the administration of labeled amino acid.

the oral administration of labeled protein or amino acid, or intravenous administration of amino acid. These three administrative techniques have the common effect of providing an immediate supply of highly labeled amino acids to all sites of protein synthesis. It makes little difference whether these amino acids enter the blood stream by direct injection or by intestinal absorption of an amino acid or digested protein meal. The plotted data obtained from endogenous labeling experiments usually follow the form shown in FIGURE 5. This curve may be divided into the following four phases:

Phase 1 represents the rapid incorporation of the amino acid radioactivity into the protein and the rapid release of newly synthesized labeled protein into the general circulation during the first twenty-four hours. Phases 2, 3, and 4 have the same interpretation as already discussed for the Hevesy labeling procedure. The major difference is the shortened duration of Phase 3 due to the fact that re-entry of isotope, represented by Phase 4, becomes significant earlier in the experiment. The greater simplicity of the endogenous procedure would make it appear to be the technique of choice if it were not for some of its serious inherent errors discussed below.

Errors and Assumptions of the Biosynthetic (Intrinsic) Labeling Procedures

Hevesy Labeling Procedure

Size of dose of protein. Although it is desirable in isotope studies to introduce the labeled substance in trace quantities sufficiently small to prevent consequent metabolic changes, this is often difficult where labeled proteins are being administered.

Immediately after giving the labeled amino acid to the donor there is a dilution of the administered radioactivity in the respective amino acid pool. As the labeled amino acid is incorporated into protein there is a further dilution of the administered isotope both by the body pool of the specific protein studied and also by all other proteins that simultaneously incorporate the administered amino acid. Thus it is expensive to infuse recipients with small amounts of donor labeled plasma proteins that have high specific activity. We have calculated that only 4 to 7 per cent of orally administered S^{35}-labeled l-cystine and approximately 1 per cent of C^{14}-labeled l-lysine was distributed as protein throughout the total circulating plasma of the mammals studied.[3, 10] In order to infuse sufficient radioactivity in the form of labeled protein into recipients, we found it necessary to increase the recipient's total rapidly circulating plasma proteins by 1.5 to 3 per cent for humans and 3.5 to 5 per cent for dogs. While such increases in plasma protein are small and may not appear to accelerate protein catabolism, these are not trace quantities and might result in shifting some of the protein metabolic equilibria.

Purity of infused protein. It is essential to administer the specific donor-labeled protein to be studied as a single molecular species, free of other proteins having the same isotope label if the apparent turnover time of the protein in the recipient is to be determined by isotopic analysis of whole

plasma, or if the size of the rapidly equilibrating pool or the entire body pool of the protein is to be calculated from the specific activities of purified samples of the recipient's protein. If a mixture of plasma proteins labeled with the same isotope is injected, the correct pool size is obtainable only if each protein species is diluted equally, if it equilibrates between compartments at the same rate, and if it is metabolized at the same rate.

Endogenous Labeling Procedure

Re-entry of the protein label. This error is a serious one in the study of endogenously labeled proteins following administration of labeled amino acid. The degree of isotope reincorporation by plasma proteins is far greater under these circumstances than when labeled proteins are injected as shown in FIGURE 6. Each of the eight turnover curves reflects both the incorporation and loss of isotope by the protein in addition to any continued equilibra-

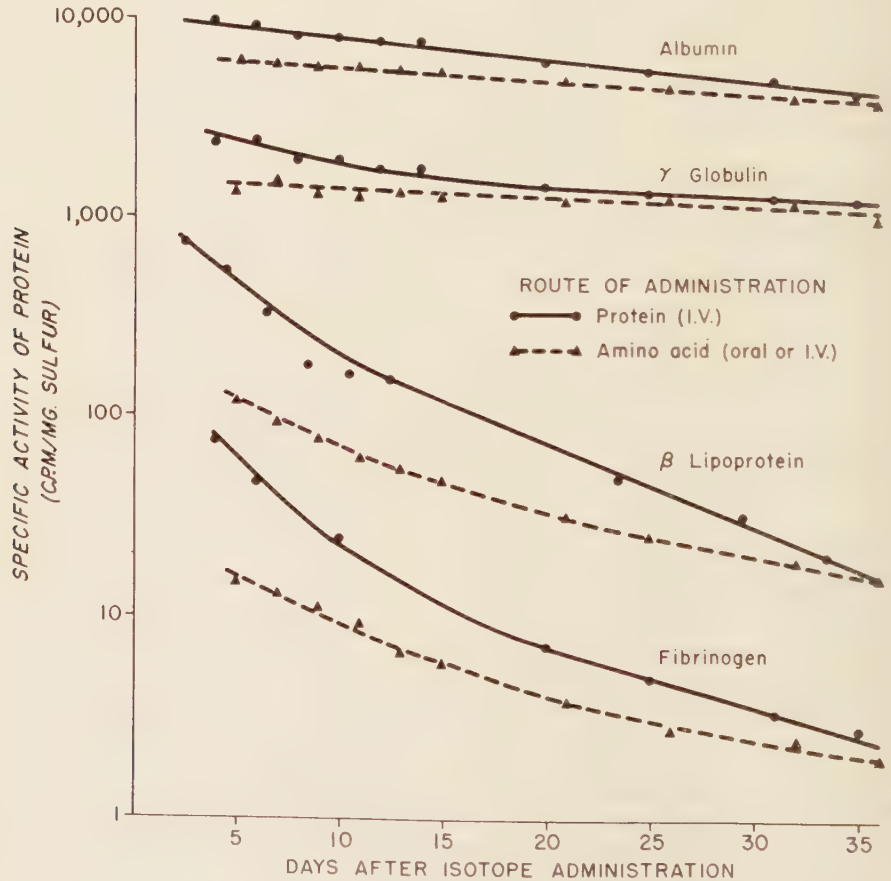

FIGURE 6. Turnover curves following the intravenous administration of S^{35}-labeled homologous human plasma proteins (solid line) to a normal subject and oral administration of labeled amino acid (broken line) to a different normal subject.

tion among the various protein spaces. The observed loss of isotope from the protein, following administration of labeled protein to a single subject, is shown by the solid curve in each case. Following administration of labeled amino acid to a separate subject, the loss of isotope from the protein is balanced to a considerable extent by a gain in isotope due to re-entry. The net result is a reduced rate of isotope loss, as shown by the flatter broken curve.

An idea of the possible magnitude of the reincorporation following injection of labeled protein can be obtained from FIGURE 7, where the distributions of radioactivity along paper strips following zone electrophoresis of recipient rat plasma are shown. In these experiments, Maurer and Müller[40] apparently have found significant incorporation of the isotope from S^{35}-labeled albumin into the α, β, and γ globulins of rats. Immediately following administration of the isotope, radioactivity of the plasma protein was located exclusively in the albumin fraction. However, approximately 18 per cent of the radioactivity present in all the plasma proteins 4 days* after administration of the isotope was located in the globulins. After 25 days this increase had risen to 68 per cent. Since attempts by McFarlane's group[42] to reproduce these observations have proved unsuccessful, 2 possible explanations exist. First, if both groups of investigators have adequately purified the administered labeled protein to remove all traces of other labeled proteins, then other differences in experimental conditions, such as the isotope used, are responsible for the discrepancy between the results obtained by the 2 laboratories. A second explanation could be that the labeled albumin preparation administered by Maurer and Müller still contained globulins. The increase in globulin radioactivity with time could then be primarily an apparent one, since it increases relative to the radioactivity of the albumin, which is degraded more rapidly than the globulins in the rat.[43] It is also possible for globulin initially distributed in the extravascular spaces to re-enter the plasma space more slowly than does albumin. The apparent absence of radioactivity in the globulins on day zero may mean either that there was an insignificant amount of labeled globulin injected along with labeled albumin, or that the labeled globulin entered extravascular spaces more rapidly than did the albumin.

A large percentage of any administered amino acid activity rapidly becomes incorporated into the proteins of all tissues. As a consequence, most body "compartments" are "isotopically enriched" with free or incorporated labeled amino acids in relatively large quantities. In animals injected with labeled proteins, however, the isotope is initially present almost exclusively

* Niklas and Maurer[41] have compared the turnover times for albumin in different species and have concluded that these values are inversely proportional to the cube root of the body weight. As a consequence, these investigators have shown that the turnover of human albumin is about 10 times slower than that for the rat. Assuming the same distribution of isotope from S^{35}-labeled albumin to occur in the human, we have estimated, from the data of Niklas and Maurer[41] that 18 per cent of the administered albumin radioactivity may be incorporated into other plasma proteins in 40 days, a lesser quantity re-entering the albumin.

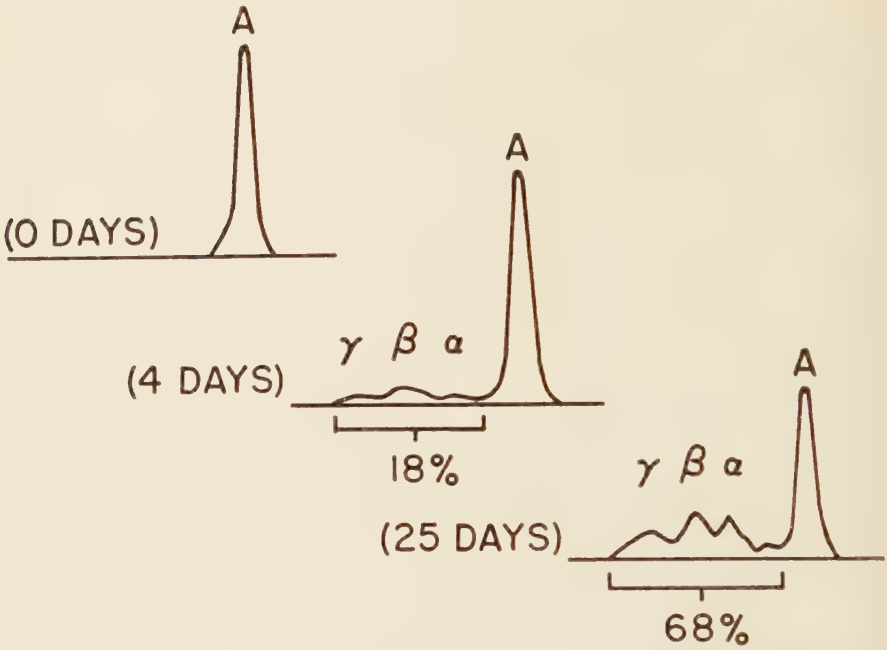

FIGURE 7. Distribution of S^{35} resulting from paper electrophoresis of plasma taken immediately, 4 days, and 25 days after the injection of homologous methionine-S^{35}- and cystine-S^{35}-labeled albumin into the rat (Maurer and Müller[40]).

in the body pool of the administered protein. In this case, most of the other body compartments are left "isotopically empty."

The extent of dilution of labeled amino acids by unlabeled ones released during the natural process of *in vivo* protein degradation, therefore, depends upon the form of the isotope administered. Dilution in isotopically empty compartments following the administration of labeled protein is relatively large, and it results in a minimal reincorporation of isotopes. Following the administration of labeled amino acids, however, the dilution in the isotopically enriched compartments is smaller, which causes a greater reincorporation of isotope.

Choice of labeling amino acid and labeling isotope. Selection of an amino acid whose labeled position is readily transferred into other amino acids would result in the simultaneous introduction of the isotope at the sites of different amino acids. Consequently, there would be a greater dilution of the administered radioactivity due to the fact that the accumulated pool sizes of several amino acids are greater than the pool size for a single amino acid. Variations in this dilution and different rates of loss and exchange of the labeled positions for different amino acids would result in different rates of isotope incorporation and reincorporation for each amino acid.[44, 45] However, if we select amino acids whose labeled positions are incorporated into

other amino acids to a negligible extent, if at all, these administered amino acids would be the only ones to label the proteins.

Studies[28] have been made of the rate of $C^{14}O_2$ expiration from rats whose plasma proteins were labeled with amino acids containing isotopes in a highly catabolized position. The rate of $C^{12}O_2$ expiration was found to be a measure of the degradation of labeled protein comparable to the reflection of the degradation of I^{131}-labeled protein found in the urinary excretion of radioiodine.[27, 28, 32, 43]

The use of singly labeled proteins to measure turnover rates is based upon the assumption that the turnover of the labeled amino acid, which is what is actually being measured, represents the turnover of the entire protein molecule in which it is incorporated. Our studies[10] with cystine-S^{35} and lysine-6-C^{14} doubly labeled dog plasma proteins (FIGURE 8) indicate an

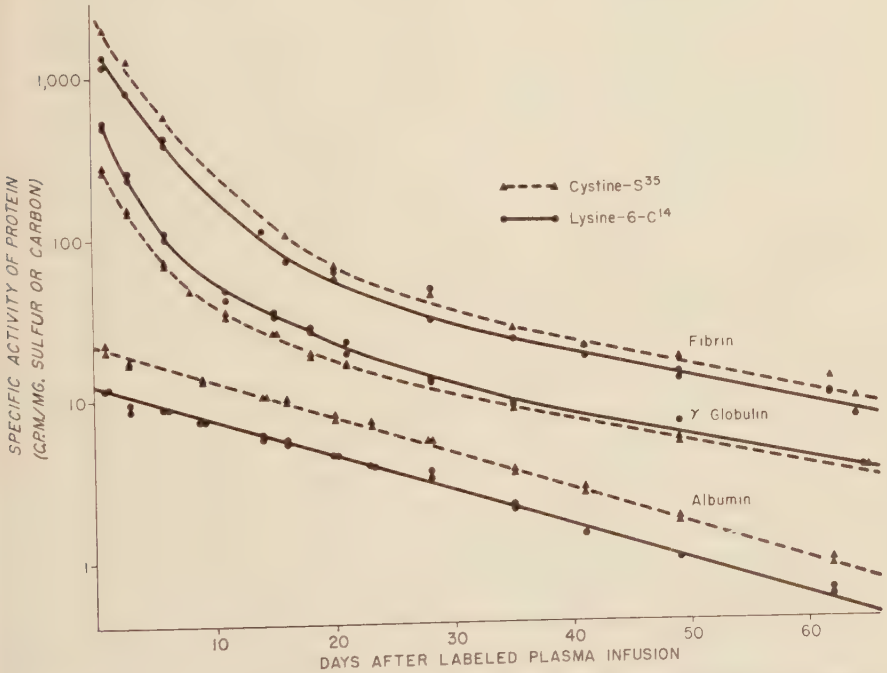

FIGURE 8. Comparison of the turnover of cystine-S^{35} (broken line) with that of lysine-6-C^{14} (solid line) in fibrin, albumin, and γ-globulin following the intravenous administration of doubly labeled homologous plasma proteins to the dog.

identical turnover of these two amino acids. However, the study of additional simultaneously labeled sites in these and other proteins would reduce the probability that amino acids chosen at random would have coincidentally equal turnover rates not equivalent to the turnover of the protein molecule, and it would help to verify the more widespread application of this assumption.

The assumption has been made that the use of the intrinsic labeling isotopes C^{14} and S^{35} permits the introduction of a tracer tag which is indistinguishable from the naturally occurring isotopes of C^{12} and S^{32}. Evidence has accumulated[35, 46, 48] to indicate that differences can be demonstrated under certain conditions. This isotope effect is greater for C^{12}-C^{14} than it is for either S^{32}-S^{35} or I^{127}-I^{131}, according to Bigeleisen,[49] who concludes that the use of C^{14} in a nonequilibrating system may require special corrections. However, since the isotope effect decreases the longer the chemical reaction is allowed to proceed, this influence will be less than is calculated.[49] The maximum isotope effect observed so far for C^{14} in *in vitro* biological reactions[46, 47] has been about 10 per cent. Consequently, the alteration of the *in vivo* measurements of most metabolic processes, including protein turnover, is probably negligible. Therefore, until it has been demonstrated that turnover measurements are sufficiently sensitive to be influenced by the substitution of unnatural isotopes, the continued use of C^{14} and S^{35} to obtain these measurements by intrinsic labeling techniques appears to be justified.

Purity of the isolated protein. Isolation of a protein mixture consisting of a large amount of the principal protein *A* and a small amount of the contaminating protein *B* will yield a *smooth nonexponential curve* if: (1) the contaminating protein *B* has a much higher specific activity than the principal protein *A*; (2) the two proteins have significantly different turnover rates; and (3) the fractionation procedure consistently yields protein preparations with the same degree of impurity as shown in TABLE 2. The smaller the pool size of protein *B* relative to that of protein *A*, the greater will be the contribution of the turnover of *B* and, consequently, the greater the deviation of the *nonexponential curve* from the turnover curve of *A*. Variation of the degree of impurity from one sample to another would result in an *erratic nonexponential curve* caused by an exaggerated scattering of the plotted data. Therefore, the reproducibility of the composition of a protein fraction is essential in work where purity of molecular species is unattainable. If both proteins *A* and *B* have essentially identical rates of turnover, the exponential turnover curve (X_A) of *A* will be observed. If proteins *A* and *B* have different rates of turnover and the specific activity of protein *A* is less than that of protein *B*, or if the pool size of protein *A* is greater than that of protein *B*, a nonexponential curve ($X_A + X_B$) will be observed.

Steady state of protein metabolism. One of the assumptions necessary for the interpretation of protein turnover data is that no change should occur in either the rates of protein synthesis or degradation during the course of the experiment. The presence of a steady state implies that the rates of synthesis and degradation of the protein are equal and that there is no net change in the quantity of protein. McFarlane[50] has pointed out that repeated withdrawals of blood from the animals, resulting in a falsely reduced turnover rate, cannot be avoided and that correction must be made for it. This is probably due to continued stimulation of "extra" plasma protein synthesis and a cumulative dilution of the labeled proteins by this extra protein, plus protein from extravascular compartments. The correction is made, according to McFarlane,[50] by increasing the specific activity of each

TABLE 2

EFFECTS UPON TURNOVER CURVES* OF RELATIVE SPECIFIC ACTIVITIES, POOL SIZES, AND TURNOVER TIMES OF PROTEINS IN A MIXTURE†

	$t_{\frac{1}{2}A} = t_{\frac{1}{2}B}$		$t_{\frac{1}{2}A} \gtrless t_{\frac{1}{2}B}$		
	$PS_A \gtrless PS_B$	$PS_A = PS_B$	$PS_A < PS_B$	$PS_A = PS_B$	$PS_A > PS_B$
$sa_A < sa_B$	X_A (straight)	X_A (straight)	X_A + slight X_B (very slight curve)	X_A + small X_B (slight curve)	X_A + X_B (curved)
$sa_A = sa_B$	X_A (straight)	X_A (straight)	X_A (straight)	X_A +slight X_B (very slight curve)	X_A + small X_B (slight curve)
$sa_A > sa_B$	X_A (straight)	X_A (straight)	X_A (straight)	X_A (straight)	X_A + slight X_B (very slight curve)

*Data plotted semilogarithmically.

†Mixture consists of a large amount of protein A and a small amount of protein B; $t_{\frac{1}{2}}$ = half life, PS = pool size, sa = specific activity, and X = exponential curve represented by the observed turnover curve (shape indicated in parentheses); subscripts A and B refer to proteins A and B, respectively.

point on the apparent turnover curve by the ratio $P/P\text{-}S$ where P is the total rapidly circulating plasma protein mass* and S is the mass of plasma protein removed at the previous bleeding. The increase in the level of plasma proteins following the administration of labeled protein and its probable effect upon increased protein degradation already has been discussed under the subheading *Size of dose of protein.*

Analysis of the plotted data. Care must be exercised in correctly locating that segment of the exponential curve that actually represents the metabolic degradation of the protein. Inclusion of part of either the initial equilibration phase or terminal reutilization phase would obviously introduce an error into the calculation of turnover times. In an experiment of too short duration it may be impossible to differentiate accurately between the distribution and metabolic phases.

Although the completion of Phase I (FIGURE 3) is generally assumed[27] to represent equilibration of the labeled protein with the total exchangeable

* The mass of the plasma protein is calculated as follows: plasma volume (ml.) × plasma protein concentration (gm./ml.) = intravascular protein mass (gm.); intravascular plasma protein mass × f = extravascular protein mass; intravascular plasma protein mass × (f + 1) = total protein mass in the total body pool of the protein; f is a factor variously estimated as being 1.5,[32] 4.5,[48] and 0.9 to 2.2[50] for human albumin; 1[50] and 1.5 to 1.7[49] for rat albumin; 1.3 to 1.5[51] for rat γ globulin; and 0.7[31] for guinea pig globulin.

pool, we and others[29, 41] believe that this equilibrium is never attained. Since the rate of exchange of plasma protein between the various fluid pools of the body is highly variable and since very slow transfers are made between some of the tissue lymph spaces and the plasma space,[32, 43] mixing proceeds continuously.

Relative Effects of Biosynthetic (Intrinsic) and Chemical (Extrinsic) Labeling Techniques

We believe that the clearest comparison of the metabolic fates of proteins labeled by means of these two techniques should be evident in experiments wherein proteins labeled by both techniques have been studied simultaneously in the same subject. Consequently, we have limited our discussion to published data from experiments of this type only. The very limited amount of data available for such comparative labeling experiments may be due chiefly to the magnitude of time and effort required.

Administration of S^{35}- or C^{14}-labeled proteins plus I^{131}-labeled proteins. Margen and Tarver[38] studied albumin degradation in 8 humans following the intravenous administration of methionine-S^{35}-labeled plasma proteins and I^{131}-labeled albumin; we[3] have studied albumin degradation in 2 humans following intravenous administration of cystine-S^{35}-labeled plasma proteins and I^{131}-labeled albumin, while Armstrong's group[26] has administered

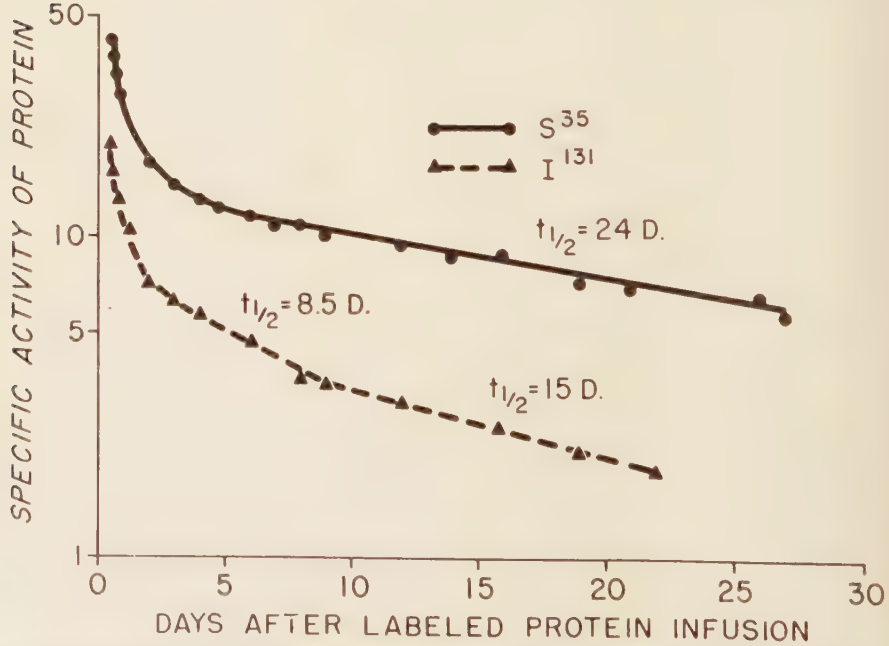

Figure 9. Simultaneous comparison of the turnover of S^{35}-donor-labeled albumin with that of I^{131}-labeled albumin from the same human subject following the intravenous administration of the labeled homologous protein (Margen and Tarver[38]).

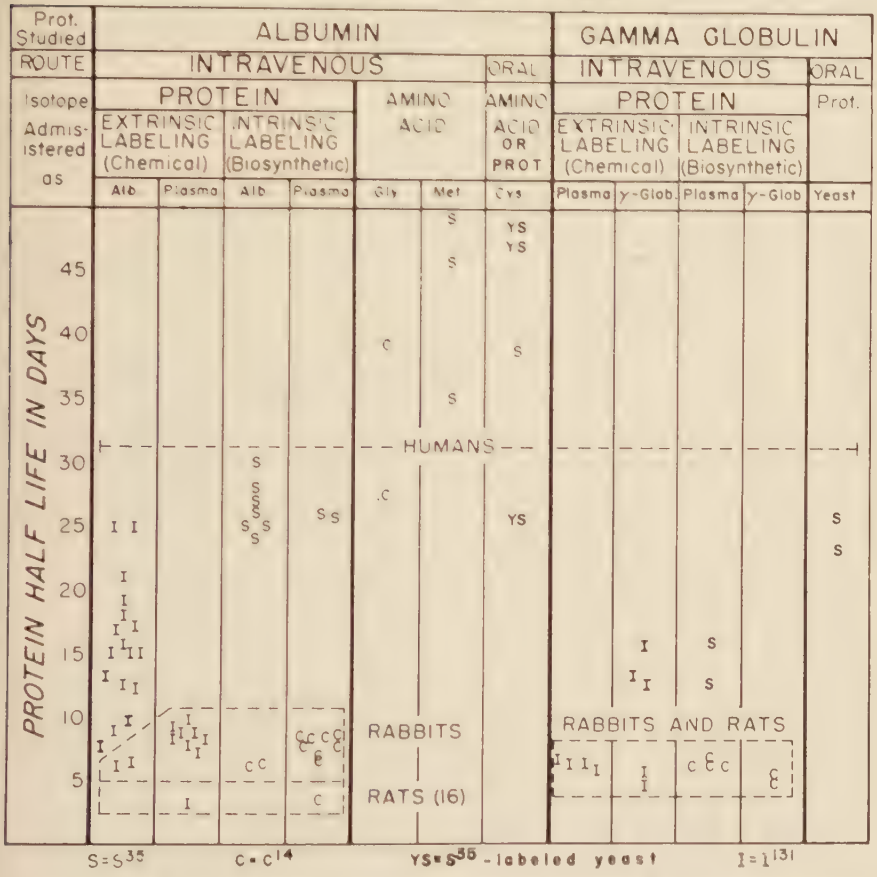

FIGURE 10. Summary of the published data on the turnover of albumin and γ globulin in the human, the rabbit, and the rat following the simultaneous administration of chemically labeled protein with biosynthetically labeled protein or labeled amino acid. For intravenous administration of C^{14}- or S^{35}-labeled proteins, the following reports are included: Margen and Tarver,[39] 8 humans; Volwiler *et al.*,[3] 2 humans; Armstrong, Bronsky, and Hershman,[38] 2 humans; Cohen *et al.*,[37] 10 rabbits; Campbell *et al.*,[43] 16 rats. For the administration of C^{14}- or S^{35}-labeled amino acids or orally given whole protein: Armstrong *et al.*,[40, 41] 5 humans; Margen and Tarver,[39] 3 humans; Masouredis and Beeckmans,[51] 2 humans; Volwiler *et al.*,[8] *1 human.*

S^{35}-labeled plasma (from a donor fed S^{35}-labeled yeast) and I^{131} labeled γ-globulin intravenously in studying γ globulin degradation in 2 human subjects.* Tarver's group and ours have both reported that in all subjects, shorter turnover times were obtained for the I^{131} labeled albumin as shown

*One subject (A.W.) received labeled plasma from a uremic donor rather than from a normal one.

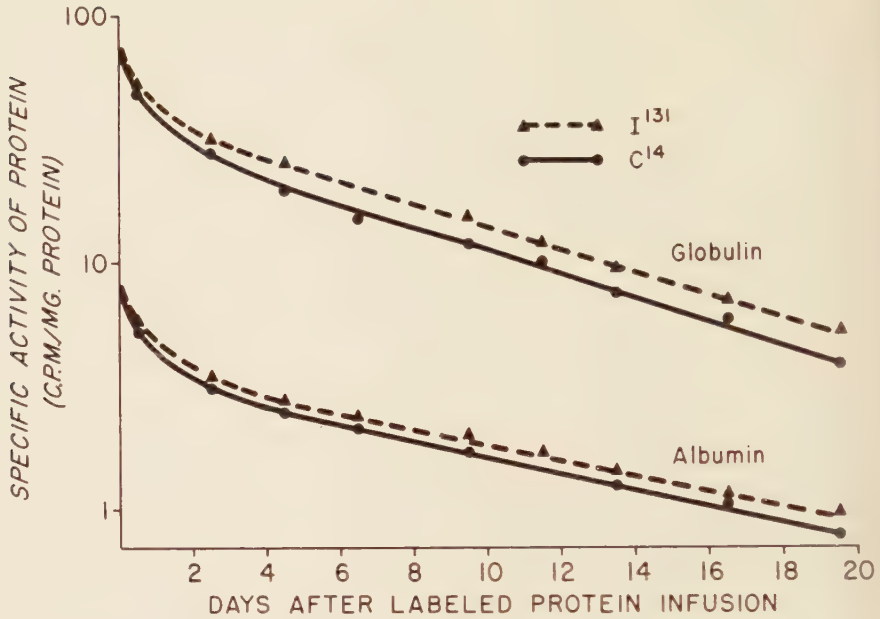

FIGURE 11. Elimination of I^{131}- and C^{14}-labeled albumin and globulin following the simultaneous intravenous administration to the rabbit of these homologous proteins (Cohen *et al.*[27]).

by a typical set of curves in FIGURE 9 and the summarized data in FIGURE 10.

In contrast, McFarlane and his co-workers[27, 28, 43] have shown that it is possible to obtain identical turnover times for both C^{14} and I^{131}-labeled albumin, γ-globulin, or fibrinogen, as shown in FIGURE 11. This was accomplished by the administration of C^{14}-labeled plasma or serum plus I^{131}-labeled albumin,* γ-globulin* or plasma in 10 rabbits and 2 groups of 10 rats and measuring the change in specific activity of the respective isolated proteins.† While the apparent half lives differed by less than 2 per cent for the 2 differently labeled proteins, there appeared to be some biological difference. A certain percentage of the I^{131}-labeled protein was removed more rapidly from the circulation, as shown by the fact that the extrinsically labeled protein exhibited a greater S_{100}[28, 40] value and a lower distribution ratio[28] than did the intrinsically labeled protein. That the success of McFarlane's group in obtaining similar turnover data for both biosynthetically and I^{131}-labeled proteins is thus far unique is shown in FIGURE 10, where the available published data on the turnover of labeled albumin and γ-globulin has been summarized.

* These administered labeled proteins contained traces of α- and β-globulins.[27]

†The isolated proteins ranged in purity from traces to 20 per cent of α- and β-globulins in the albumin and γ globulin and included some γ-globulin samples which contained 4 per cent albumin.[27, 28, 43]

Administration of S^{35}- or C^{14}-labeled amino acid plus I^{131}-labeled protein. Albumin degradation was studied by Margen and Tarver[35] in 3 humans following the administration of S^{35}-labeled methionine and I^{131}-labeled albumin; by Masouredis and Beeckmans[51] in 2 humans following administration of glycine-2-C^{14} and I^{131}-labeled albumin (FIGURE 12); and in our

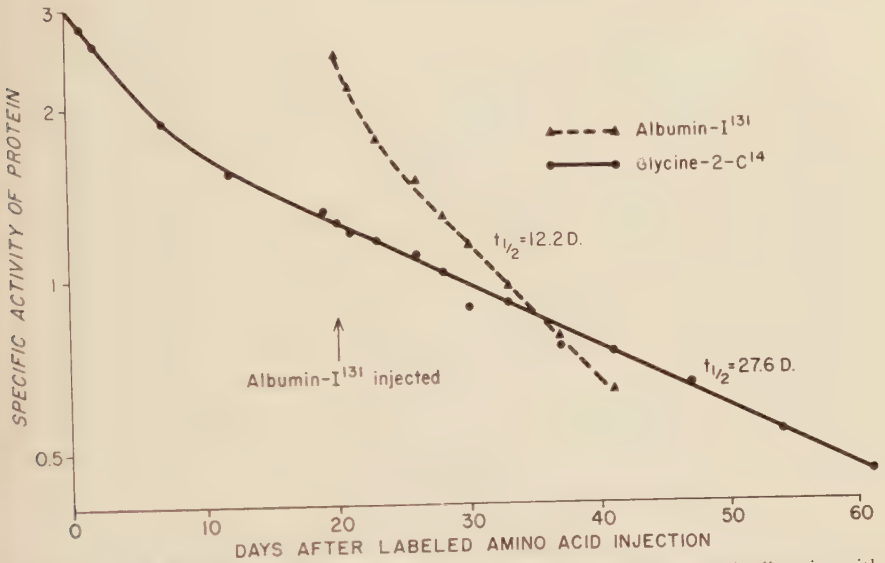

FIGURE 12. Simultaneous comparison of the turnover of C^{14}-labeled albumin with that of I^{131}-labeled albumin in a patient with polycythemia vera following the intravenous administration of glycine-2-C^{14} and human albumin-I^{131} (Masouredis and Beeckmans[51]).

laboratory[4] in 1 human following the administration of S^{35}-labeled cystine and I^{131}-labeled albumin. Armstrong and his group[26, 52] have reported 3 cases* where the turnover of γ-globulin was studied in humans following the oral administration of S^{35}-labeled yeast and the injection of I^{131}-labeled γ-globulin, and 3 cases[53] where the turnover of albumin was studied in humans following the oral administration of S^{35}-labeled yeast and the intravenous administration of I^{131}-labeled albumin. In each instance the turnover rate observed with I^{131} was faster than that obtained with the C^{14}- or S^{35}-labeled amino acids (FIGURE 10).

Conclusions

(1) There is a very limited amount of data, published by only a few authors, describing the employment of the biosynthetic Hevesy labeling procedure. This is undoubtedly due to the complexity and expense of the intrinsic labeling technique.

(2) American investigators have observed consistently faster turnover

* We believe the data presented for subject G.B.[26] to be inadequate for inclusion in the summary in FIGURE 10.

rates in the same animal for I^{131}-labeled proteins than for C^{14}- or S^{35}-labeled proteins. This appears to be due chiefly to alterations in the I^{131}-labeled protein occurring during the isolation, iodination, and handling processes prior to its administration.

(3) McFarlane's group is the first to have iodinated proteins without changing their apparent turnover rates relative to those for corresponding C^{14}- or S^{35}-labeled proteins. The simplicity and effectiveness of their methods should make it possible to avoid the problems raised in (1) and (2).

(4) Slower turnover rates have been obtained following endogenous labeling with amino acids, compared to those observed with the Hevesy labeling procedure. This is due to the far greater reutilization of isotope following labeled amino acid administration.

(5) It is permissible to select a labeling procedure that may alter the protein, provided that any immediate or secondary changes in properties, functions, or processes to be measured cannot be detected. While most iodinated albumin appears adequate for blood volume and immunochemical determinations, and the unique iodination technique of McFarlane's laboratory appears to be sufficient for measuring metabolic turnover rates, it is conceivable that future investigations of increased sensitivity, such as detailed studies of enzyme and other metabolic mechanisms, may require exclusive use of even the mildest intrinsic labeling technique.

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THE DEIODINATION OF PROTEINS LABELED WITH I^{131}*

By Sheldon Margen and H. Tarver
Department of Physiological Chemistry, University of California School of Medicine, Berkeley, Calif.

During the past few years a great number of metabolic studies have been done with proteins labeled with I^{131}, on the assumption that the loss of iodine from the protein is a measure of its degradation. Generally, it is assumed without question that iodine is lost neither by exchange nor by any reaction that does not involve the rupture of the peptide bonds. The purpose of this paper is to call attention to the fact that this assumption probably is erroneous, at least for some experimental arrangements. In particular, this assumption is certainly incorrect for human serum albumin iodinated by the method noted below when this material is used in studies of turnover. This is shown readily by using serum albumin doubly labeled with methionine-S^{35} and I^{131}. In one instance this same phenomenon has been observed by the use of human serum albumin that had been biosynthetically labeled with S^{35} and iodinated with I^{131}.

Samples of human serum albumin† were labeled with L-methionine-S^{35} by polymerizing the amino acid on to the protein by methods developed from those employed by Farthing[1] and Stahmann,[2a, b, c] in such a way as to produce products with 1 to 12 mol of methionine per mol (65,000) of albumin.

As an example, the material (preparation 3A) given to patient Pea. (FIGURE 1, TABLE 1) was prepared as follows: 30 mg. of methionine-S^{35} was placed in a dry closed vessel into which was distilled approximately 4 ml. of dioxane (from sodium). The temperature was then raised to 80 to 90° C., and a slow stream of phosgene was admitted over a period of 15 min., although, after about 5 min., all of the methionine had apparently reacted; that is, all of it was in solution. Excess phosgene was caught in a suitable trap arrangement. The pressure was then lowered, allowing the dioxane, together with any dissolved phosgene, to be distilled off. More dioxane was added and removed 3 times. The final product was dissolved in 4 ml. of fresh dioxane. Two ml. of this methionine carbaminoanhydride reagent was added to an ice-cold solution of 2.5 gm. of human serum albumin dissolved in 25 ml. of 0.2 *M* phosphate buffer at *p*H 7.0 while the solution was rapidly mixed with a magnetic stirrer. Some of the albumin precipitated at this point, but it returned into solution later when more water was added. The mixture was allowed to stand for several hours in the refrigerator and then was subjected to dialysis for 2 days in the cold room, first against acetate buffer at *p*H 4.6, and finally against water. The yield on the basis of determi-

* The work reported in this paper was supported in part by a grant from the National Cancer Institute, Public Health Service, Bethesda, Md.

† We are indebted to Cutter Laboratories, Berkeley, Calif., for supplies of Fraction V from human serum albumin prepared by the Cohen method.

nation of radioactivity on the dialyzed preparation was 60 per cent of the original activity in the methionine, and of this 96.2 per cent was precipitable with trichloroacetic acid (TCA); that is, 58 per cent of the original 15 mg. of methionine was actually bound, or approximately 1.5 mol of methionine were fixed per mol of albumin. When the amount of albumin was reduced to

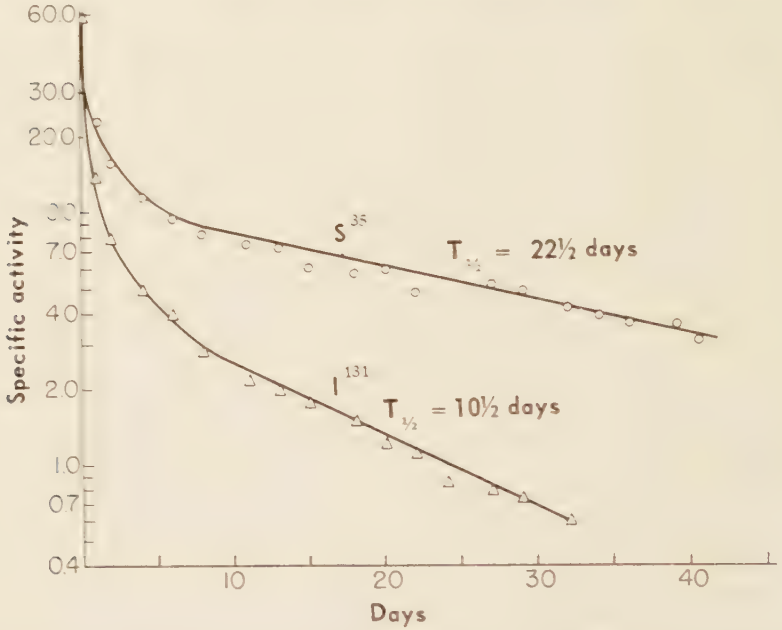

FIGURE 1. The relationship between the specific activity of I^{131} and S^{35} in the serum and time in a patient (Pea.) who had received an injection of human serum albumin labeled *in vitro* with methionine-S^{35} and I^{131} (AM-35-131).

The specific activity is plotted on a logarithmic scale in arbitrary units so that the zero time values coincide. The method of preparation of the labeled albumin is given in the text.

TABLE 1

PREPARATION OF ALBUMIN LABELED WITH METHIONINE-S^{35}

Date	No.	Albumin	Methionine-S^{35}	Methionine-S^{35} bound	Ratio*
		gm.	mg.	per cent	
Jan. 1955.....	3A	2.5	14.7	58†	1.5
Jan. 1955.....	3B	0.25	14.9	46†	12.0
Apr. 1956.....	4A	2.0	12.6	74‡	2.0
July 1956.....	4B	0.20	8.0	30‡	5.3

*Mols of methionine-S^{35} bound per mol of albumin (65,000).
†Condensation in phosphate buffer 0.2 *M*, *p*H 7.0, 25 ml.
‡Condensation in bicarbonate solution 5 per cent, 50 ml.

250 mg. the methionine fixed was somewhat less, namely, 40 per cent. Carrying out the coupling reaction in 5 per cent bicarbonate solution did not appear to improve the yield significantly. Lower yields were obtained when less methionine was employed.

The dialyzed solution was centrifuged to remove traces of denatured albumin, yielding a product as clear as water. When subjected to zone electrophoresis on paper, the modified albumin (AM-35) moved, together with its label, at essentially the same rate as normal serum albumin when they were run simultaneously in barbital buffer at pH 8.6 and ionic strength 0.05. The preparation therefore contained no large TCA-precipitable methionine peptides.

The AM-35 so prepared presumably had most of the methionine attached to the free ε amino groups of the lysine residues because, in this protein, there is a ratio of 55 such groups to the single N-terminal amino group.[3] However, this problem has not been investigated specifically.

The plasma albumin AI-35 labeled internally with S^{35} was prepared in a manner similar to that described by Margen and Tarver.[4] The only modification was that the albumin was isolated by the Cohn X fractionation procedure performed in our laboratory. The albumin so obtained was found to be approximately 90 per cent pure by electrophoretic analysis. The AM-35 and AI-35 were then iodinated by a modification of the method of Gilmore and his co-workers.*[5] Carrier iodine was added in an amount sufficient to provide 3 to 4 atoms of iodine per mol of albumin. The doubly labeled material was again subjected to dialysis to remove any free iodine or iodide. The final products AM-35-131 and AI-35-131 contained less than 5 per cent of the I^{131} free in solution after precipitating the albumin with TCA. The solution was then sterilized by filtering through a Seitz filter.

Several specimens of AM-35-131 and 1 sample of AI-35-131 were injected into patients hospitalized for conditions unassociated with abnormalities in protein metabolism. During 4 or more weeks following the injection, the radioactivities in samples of blood and serum were followed; the I^{131} with a scintillation detector of the well type, and the S^{35} by procedures involving digestion and conversion of the protein sulfur to benzidine sulfate. The radioactivity of the sulfate was measured on paper in the usual way with a Geiger-Müller counter, and its amount was determined by titration.[4]

The results are summarized in TABLE 2, and the results of a typical case in which the patient was given 1.01 gm. of the AM-35-131 preparation described above are shown in FIGURE 1. This illustrates the behavior in time of specific activity of the label, either S^{35} or I^{131}, expressed in arbitrary units adjusted so that the initial specific activities of both have the same value. From these data it is clear that the I^{131} was lost from the protein at a much more rapid rate than was the S^{35}, and that this difference in rate persisted over the whole period of the experiment. These results were dupli-

* The method was modified by the substitution of potassium persulfate (0.15 M) in buffer for the ammonium persulfate (0.44 M) used by these investigators. The buffering proved to be ineffective with ammonium persulfate; moreover, this concentration appears to be stronger than necessary.

TABLE 2
METABOLISM OF ALBUMIN

Subject	Age and sex	Diagnosis	Preparation No.	Preparation Dosage	Label S^{35} Longest 1/2 life	Label S^{35} Observation	Label I^{131} Longest 1/2 life	Label I^{131} Observation
				mg.	days	days	days	days
Pea.	54 F	Paraplegia	3 A	1036	22.5	41	10.5	32
Ses.	33 M	Neurofibromatosis	4 A	870	24	50	10	19
Jef.	48 M	Poliomyelitis	4 A	870	19	50	9.5	26
Sim.	74 M	Hemiplegia	4 B	63	17	34	10	34
Gen.	42 F	Multiple sclerosis	4 B	63	15	39	10	39
Bro.	22 M	Fractures of lower leg	4 B	63	18	39	10	39

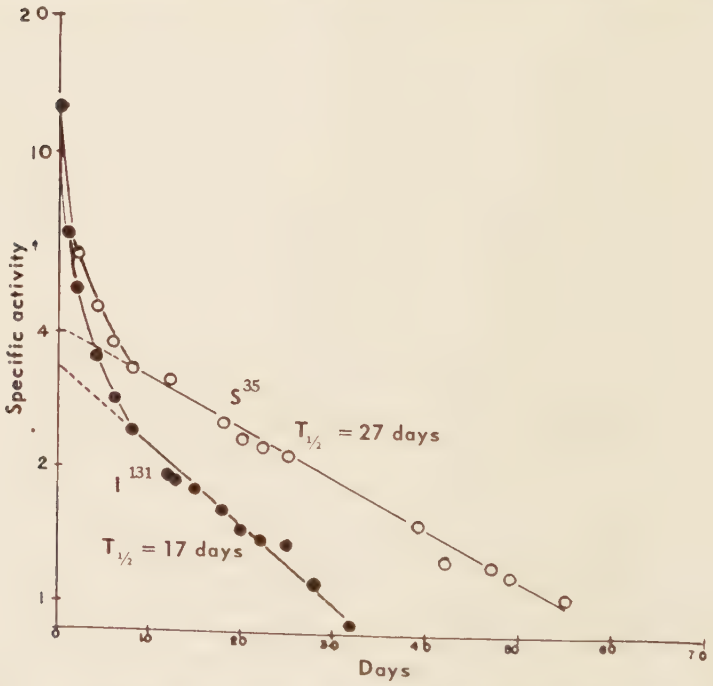

FIGURE 2. The relationship between the specific activity of I^{131} and S^{35} in the serum and time in a 68-year-old patient (Ske.) who received an injection of human serum albumin labeled *in vivo* with S^{35} amino acids and I^{131} (AI-35-131). The diagnosis of the case is old ununited fractures of the lower legs. No disturbance of the protein metabolism is apparent.

As in FIGURE 1, the specific activity is plotted on a logarithmic scale in arbitrary units so that the zero time values coincide. The method of preparation of the labeled albumin is given in the text.

cated in 6 additional subjects given the same or different preparations of AM-35-131. The results obtained when a patient was given 5.00 gm. of the AI-35-131 preparation are shown in FIGURE 2. It is clear that in this instance also the I^{131} was lost from the protein at a much more rapid rate than was the S^{35} label. Thus, although the rates of catabolism of AM-35-131 may be normal, or faster or slower than normal, the I^{131} was lost more rapidly than was the S^{35}. The same phenomenon is observed where the label is an integral part of the protein.

The amount of labeled methionine introduced into the patients in the experiments just described was small relative to the total labile methionine. The methionine was attached in traces to only one protein, and was not present as a label in the total mass of labile protein in the subjects. The results obtained from these patients should be compared with those in which methionine-S^{35} was administered.[4, 6] In the latter case an extremely long half life was found for the plasma proteins because of the reutilization of the labeled amino acid.

In the present experiments the reutilization of the labeled amino acid with high specific activity was virtually impossible, since the dilution of the methionine-S^{35} arising by the breakdown of the protein injected by the pool of free methionine must have been very high. This interpretation of the results would be in error if the site of degradation and synthesis were within the same cell, and if reincorporation were possible before the labeled amino acid liberated by the degradation could be catabolized or diluted by unlabeled amino acid in the pool. This phenomenon has not been demonstrated clearly in any system to date, although some workers have interpretated their data on such a basis.[7]

In addition, it is unlikely that the mere addition of methionine to the free amino groups of the albumin in the case of AM-35 would have any tendency to effect labels on the iodide in the tyrosine residues. This is demonstrably true, since similar results were observed in the case of the doubly labeled protein AI-35-131.

Furthermore, the partial denaturation of the protein by iodination would not effect the deductions made from these experiments. Were the protein partially denatured, the effects of denaturation would be equally apparent with both labels, since they exist together in the same molecules.

Accordingly, from these experiments we are forced to conclude that I^{131} was lost in part from the albumin by a mechanism that did not involve the rupture of peptide bonds, because this would have resulted in the freeing, solubilization, and loss of methionine-S^{35} or the S^{35} label. These findings substantiate our previous conclusions[4] and the observations of Volwiler and his co-workers.[6]

Neither of the half lives found with the preparation of AM-35-131 necessarily bear any relation to the real half life of the albumin in the subject. We are in the process of determining the relationship between the half lives observed with AM-35 and AI-35; that is, whether the true half life can be measured with the artificially labeled protein.

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Discussion of the Paper

J. L. Steinfeld (*National Cancer Institute, Public Health Service, Bethesda, Md.*): Before discussing the paper of Margen and Tarver, I should like to comment upon an issue raised by Kenneth Sterling of the University Hospital of the Good Shepherd, Syracuse, N. Y. This investigator reports that, in his experience, the half time of I^{131} albumin in normal males is closer to 11 days than to the 17 days that Berson and Yalow found in their control subjects. The latter investigators used a preparation of iodinated albumin different from that used by Sterling, and this, of course, might account for the variance in their findings.

Another possibility or contributing factor, however, is that control human subjects who have had a chronic disease, but no apparent hypoalbuminemia may, indeed, have longer half times for I^{131}-albumin degeneration than might be the case with healthy normal male volunteers. Using radioiodinated human serum albumin (RISA) in all cases, we have studied healthy young male conscientious objectors in contrast to other control subjects without hypoalbuminemia, but with a variety of chronic diseases, and we have found the half times for I^{131} albumin to be shorter by several days, on the average, in the conscientious objectors as compared with control subjects with chronic diseases.

Because of limitations of space, Margen and Tarver have been unable to describe all of the various permutations and combinations of this exciting new double *in vitro* labeling technique. The possibilities for the use of the technique are many and, indeed, its use may provide answers to questions long asked about the behavior of albumin labeled *in vitro* with I^{131} and I^{127}. The interpretation of the experiments presented by these authors is both guarded and conservative, yet implicit in any interpretation is a theory regarding, if not the synthesis, the breakdown of albumin: namely, is albumin broken down all at once into its constituent amino acids and small peptides, or is this protein broken down selectively, with more or less immediate utilization of some of the breakdown products for the synthesis of albumin?

It is possible, but I am not sure how probable, that lysine residues them-

selves are either components of albumin or parts of such components that are saved and reused, whereas the tyrosine residues (or at least the iodine in them) are neither such components nor parts of them.

If one were to subject albumin that had been labeled with S^{35} *in vitro* to an iodinating solution that one would expect to alter the albumin to such an extent that, *in vivo*, it would be degraded and excreted in a few days, one could then determine whether, in this definitely altered albumin, the S^{35} label did or did not disappear at the same rate as the I^{131} label. This, then, would give some limited insight into the theoretical problem of the piecemeal versus the all-or-none synthesis and breakdown of albumin.

RADIOCHEMICAL AND RADIOBIOLOGICAL ALTERATIONS OF I^{131}-LABELED PROTEINS IN SOLUTION

By Solomon A. Berson and Rosalyn S. Yalow

Radioisotope Service, Veterans Administration Hospital, Bronx, N. Y.

Previous experience with I^{131}-labeled proteins has frequently raised questions regarding their validity as tracers for the respective native proteins in biological studies. Unpredictable behavior in biological systems has been encountered in certain instances. For instance, it has been observed that the method of iodination of serum albumin may influence significantly its rate of metabolic degradation *in vivo* in man[1] and in the rabbit.[2] Similarly, the rate of disappearance of protein-bound I^{131} from plasma following intravenous administration of insulin-I^{131} showed a variability, at times, that is apparently unrelated to biological factors.[3] The latter observations were correlated with the presence of I^{131}-labeled components that were bound to the plasma proteins. Attempts to indict the individual chemical substances employed in the iodination procedure as the agents responsible for the production of these plasma protein-binding fractions of insulin-I^{131} were unsuccessful.[3] It was subsequently appreciated that the extent of contamination of insulin-I^{131} with these components was related to the time elapsed after preparation[3] and to the concentration of I^{131} present. It thus appeared that the total dosage of radiation absorbed by the solutions might be a critical factor, and this possibility was confirmed in experiments employing external sources of irradiation.[4] It has been known for some time that proteins are readily susceptible to the effects of ionizing radiation; particularly in the case of serum albumin, Barron and his co-workers[5] have shown changes in ion-binding capacity, spectral absorption in the ultraviolet range, and amino acid composition following X irradiation. These data should, perhaps, have already stimulated a formal investigation of the effects of radiation in proteins labeled with radioisotopes but, in the absence of obvious denaturation or other alterations, it has not always seemed necessary to probe for every conceivable source of injury to the proteins. However, it has been observed that, even though the labeled protein may appear to be identical to the native species by a number of physicochemical criteria, such as ultracentrifugal sedimentation and electrophoretic mobility, its biological behavior may be altered completely.[1] The studies on insulin-I^{131} and albumin-I^{131} described in this paper were therefore oriented toward reactions in both biological and chemical systems. While the former may be more discriminatory, elucidation of some of the chemical changes aids in interpreting anomalous biological behavior.

In some respects, at least, the nature of the alterations produced by irradiation of the labeled proteins solutions with X rays and with the self-contained I^{131} are similar.[3, 6] Therefore, most of the radiation experiments described here were performed with X rays since, aside from considerations

of the cost of I^{131} and the inexpediency attending its use, the radiation-induced liberation of iodide^{131} from the iodoproteins could not be studied by this means. I^{131}-labeled proteins with a relatively low specific activity (about 100 μc. mg.) were freshly prepared for these experiments so that alterations due to self-irradiation would be minimized. A General Electric X-ray machine, peak energy 1 Mev, was employed, and irradiated solutions received 650 to 1500 rad min.* as determined with a Victoreen ionization chamber.† As a basis for comparison, it may be noted that 10,000 rad and 50,000 rad are approximately equivalent to the radiation absorbed in 1 and 6 days, respectively, by I^{131} solutions containing 1 mc./ml.

Since the sensitivity to irradiation varies among different proteins and is dependent upon the specific reaction under study, it is important to establish the quantitative aspects of radiation injury as well as the nature of the manifestations of such injury as they relate to the biological behavior of the labeled proteins. It is necessary to review first some of the methods employed in these investigations. In the case of insulin-I^{131} particularly, paper electrophoresis and water-flow chromatography were used to evaluate some of the qualitative effects of irradiation. Chromatography is effected by permitting evaporation of water from the paper strips during electrophoresis.[3] Under the influence of the concentration of buffer salts so produced, water is drawn up osmotically from both vessels and promotes movement of the solutions toward the midline. Thus, if application is made at a site near the cathode vessel, anionic substances are moved toward the anode by both hydrodynamic and electrophoretic forces. After about 1 hour's run, unaltered insulin-I^{131} remains along the line of application‡ ("origin") whether or not plasma is present;[3, 7] the plasma proteins have moved about 2 to 2½ inches away from the origin; and any iodide^{131} that may be present has moved about 1 to 3 inches beyond (FIGURE 1). During these short runs, the movement of serum proteins due to electrophoresis alone is virtually negligible, so that they migrate as a single group primarily under the influence of hydrodynamic forces.

Following irradiation, at least two readily detectable alterations are observed:[4] the release of iodide^{131} and the appearance of labeled components that migrate with the serum proteins. The chemical nature of these components has not been established, and it appears likely that they represent a heterogeneous group of substances. On short-run chromatoelectrophoresis, a fraction of these is readily washed off the paper by water or by methanol-acetic acid after heat coagulation of the proteins and is dialyzable. Others of these labeled components migrate indiscriminately with all the serum pro-

* The rad is the unit of absorbed radiation dose, and it represents 100 ergs absorbed per gram of material. The kilorad equals 1000 rad.

† In a few experiments a dose rate of 50 rad/min. (X rays) or of 15 rad/min. (radium γ rays) was delivered to the protein solutions. The results observed were not significantly different from those observed at the higher dose rates.

‡ If the amount added is greater than 1 to 2 μg., the available binding sites on the paper become saturated at the site of application, and the tagged insulin is moved along a variable distance depending on the amount present. In contrast, the plasma proteins are not effective competitive inhibitors for the same paper binding sites.

FIGURE 1. Paper radiochromatoelectrophoretograms of control and X-irradiated solutions of insulin-I^{131} in water and in plasma before and after staining and washing in methanol-acetic acid (9:1). Insulin concentration is 20 μg. ml.; the dose of irradiation is 75 kilorad. In this figure and in FIGURE 5 the paper strips were inadvertently cut short before being photographed.

teins (as observed on prolonged electrophoresis); they are not removed from the paper by washing after heat coagulation; and they appear to be bound to the serum proteins, as indicated by their slow rate of disappearance from plasma *in vivo*.[3]

In addition to the probable heterogeneity of the labeled components migrating with the plasma proteins, it also appears as if some of the material adsorbed to the origin may likewise represent altered material, since a variable fraction washes out in methanol acetic acid while unaltered insulin-I^{131} is resistant to such treatment.

In some experiments, water flow chromatography alone was utilized. In these studies, iodide^{131} ran somewhat behind the serum proteins and was not readily distinguished from the other altered fractions.

In biological studies with I^{131}-tagged insulin, the dialyzable substances are not likely to interfere unless they are allowed to accumulate prior to use. However, as already indicated, components that bind to serum proteins can

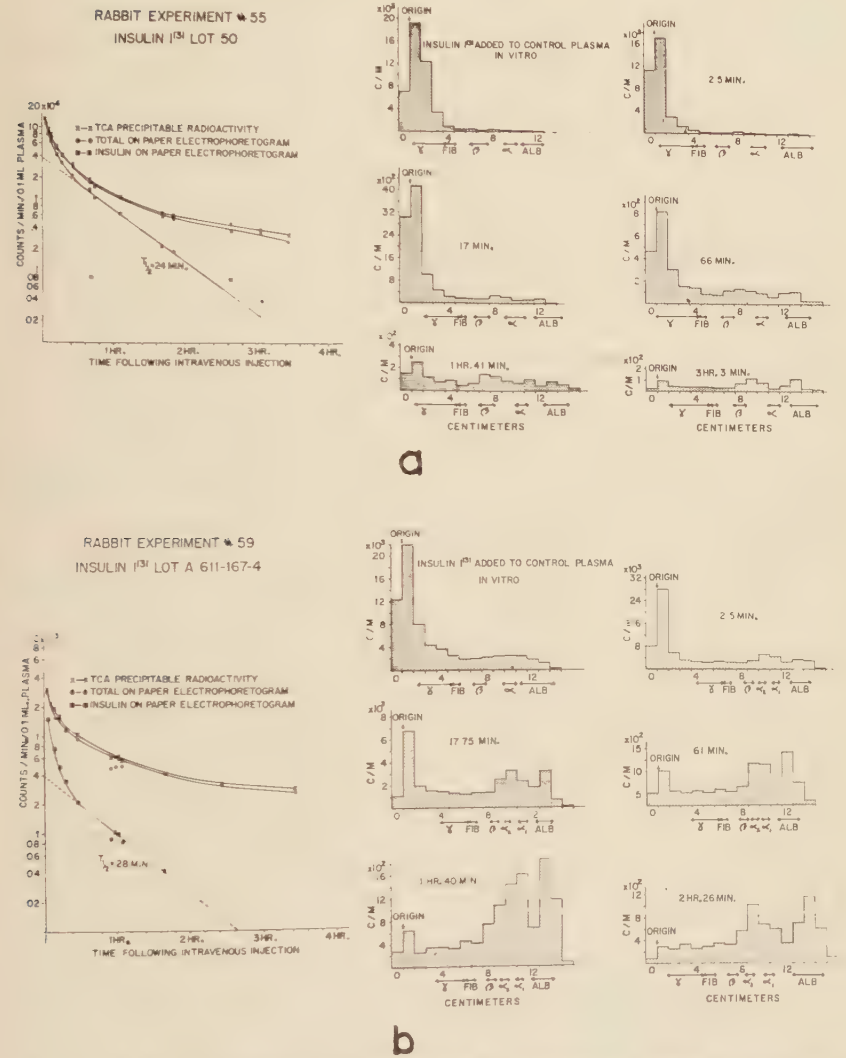

FIGURE 2. Paper radioelectrophoretograms of plasma and curves of trichloracetic acid-precipitable plasma radioactivity, total radioactivity on paper strip, and radioactivity adsorbed to paper at origin (insulin I^{131}) as a function of time following intravenous administration of two different lots of insulin-I^{131} to rabbits. (a) Preparation of insulin-I^{131} that contained a small fraction of radioactivity migrating with the plasma proteins. (b) Preparation of insulin-I^{131} that contained a relatively large fraction of radioactivity migrating with the plasma proteins. Reproduced by permission from *The Journal of Clinical Investigation*.[3]

be troublesome. Following intravenous administration of insulin-I^{131}, which was exposed only to self-irradiation from I^{131} and not to X irradiation, the form of the plasma-disappearance curves was not readily explicable in terms of the distribution and metabolism of a homogeneous substance.[3] Examina-

tion of the precipitable radioactive fractions in plasma as a function of time following administration revealed that this anomalous behavior was explained by the presence of at least 2 components in the administered material: the major one adsorbed at the origin like unaltered insulin-I^{131}, the other bound to the plasma proteins and disappearing relatively slowly (FIGURE 2). Changes were also observed in the salting-out characteristics of the plasma protein-bound radioactivity with time, which were in agreement with the electrophoretic evidence of heterogeneity of insulin-I^{131}. Since insulin itself is distributed widely and is metabolically degraded with a half time of the order of 20 to 30 min., while the plasma protein-bound material has a relatively long intravascular life span, most of the precipitable radioactivity may, even after about 30 min., represent, primarily, radiation-altered material bound to plasma proteins.[3] The extent of contamination by these altered fractions depends, of course, on the time following administration and on the amounts that are altered in the administered preparation. The important point is that if reliance is placed solely on protein-precipitable radioactivity in plasma or tissue, one may be following the fate of radiation-altered fractions rather than of insulin itself.

To proceed now to some of the quantitative aspects of radiation damage, it is evident from FIGURE 3 that the extent of alteration produced by irradia-

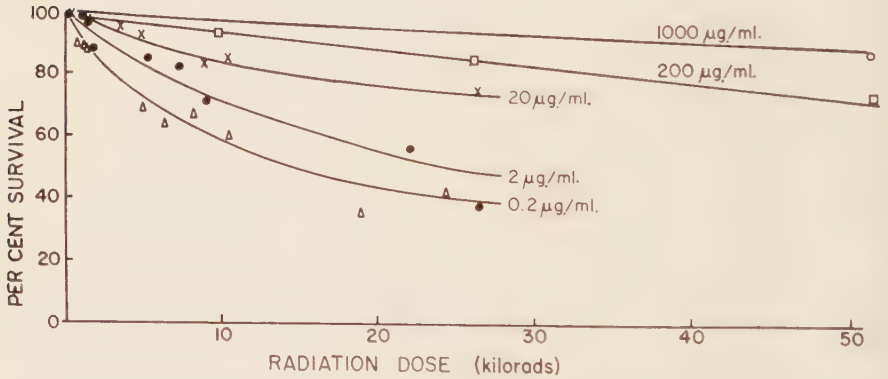

FIGURE 3. Alteration of insulin-I^{131} by X rays as a function of dosage of radiation and concentration of insulin.

tion depends not only on the dose of radiation, but also on the concentration of labeled protein; the lower the concentration, the larger the percentage of altered material.[4] However, the absolute quantity altered as a function of concentration increases to the limits of solubility of insulin with a decreasing slope (FIGURE 4). The yield (molecules altered/100 eV. absorbed) in these experiments was calculated from the total fraction that failed to bind to the paper at the origin, and includes iodide as well as components that migrate with serum proteins or that bind to them. However, it

should be emphasized that, as with other similar studies, what is being measured is simply the survival of a single characteristic: in this case, the ability to bind to paper. A considerable fraction of the radiation energy may be absorbed in excitation or ionization processes that produce either no permanent alterations or alterations that are not detectable by the specific test for survival. Furthermore, it is conceivable that radiation energy may catalyze exergonic chemical reactions. Therefore, it is common practice at

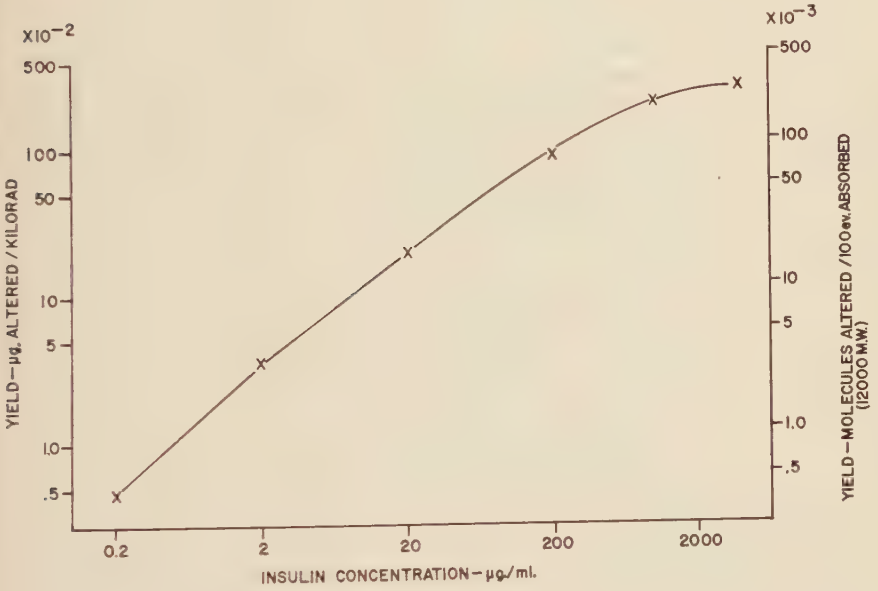

FIGURE 4. Yield of altered insulin-I^{131} as a function of insulin concentration (25 kilorads irradiation with X rays—peak energy of 1 Mev).

the present time to quantitate yield in terms of alterations produced per unit of energy absorbed rather than specifically of the energy required to produce an ion pair.

Before proceeding further, it should be noted that *p*H effects may be of considerable influence.[4] While in the case of albumin-I^{131} there appears to be no significant difference in yield of iodide-I^{131} over the *p*H range 2.65 to 9.0, the sensitivity of insulin-I^{131} to radiation decreases progressively over this range.[8]

From (1) the dependence of yield on concentration, (2) the observation that similar alterations are not produced with frozen or dried preparations of insulin-I^{131} (at similar dose levels), and (3) the virtually complete protection that is afforded by the presence of a variety of substances in the irradiated solutions,[4] it was concluded that the alterations produced are not due to a direct effect of irradiation, but that they result, rather, from the

indirect (secondary) effect of radicals formed during the irradiation of water. These processes may be summarized briefly as follows:[9]

$$H_2O \rightarrow H_2O^+ + e \quad (1)$$

$$H_2O^+ \rightarrow \cdot OH + H^+ \quad (2)$$

$$e + H^+ \rightarrow \cdot H \quad (3)$$

$$e + H_2O \rightarrow H_2O^- \rightarrow H\cdot + OH^- \quad (4)$$

In addition to the H· and ·OH radicals resulting from ionization, the excitation of water molecules may lead to direct formation of H· and ·OH along the radiation track; in this case, however, recombination occurs much more readily than when the radicals are produced at some distance from each other as following ionization.[10]

The following subsequent reactions are presumed to occur in the presence of O_2:

$$H\cdot + O_2 \rightarrow \cdot O_2H \quad (5)$$

$$\cdot O_2H + \cdot H \rightarrow H_2O_2 \quad (6)$$

$$\cdot OH + H_2O_2 \rightarrow H_2O + \cdot O_2H \quad (7)$$

Because of the various recombinations that occur (that is, $H\cdot + H\cdot \rightarrow H_2$; $H\cdot + \cdot OH \rightarrow H_2O$), the absence of precise quantitative data on the rates of production and life spans of the different radicals, uncertainty as to the effects of dissolved solutes on the relative proportion of these radicals, and the influence of such diverse factors as contamination with catalytically active trace metals and variations in pH, the total effect is quite complicated. However, it is generally agreed that the net result is the production of H·, ·OH, and $\cdot O_2H$ radicals and H_2O_2 in aerated solutions, while the production of $\cdot O_2H$ and H_2O_2 is markedly limited in the absence of O_2.

Since the irradiation of insulin in a nitrogen atmosphere uniformly leads to a marked increase in the release of iodide^{131} (FIGURE 5), it would appear that $\cdot O_2H$ and H_2O_2 are not responsible for this effect.[11] Furthermore, even high concentrations (1½ per cent) of H_2O_2 not only fail to produce these changes,[4] but are actually quite protective against them, possibly through an effect on removing ·OH radicals, as in EQUATION 7.

The possible role of ·OH radicals in the production of some of the radiation induced alterations in insulin I^{131} is further suggested[11] by the similar pattern of damage observed following treatment of insulin-I^{131} with ·OH radicals produced chemically with Fenton's reagent ($Fe^{++} + H_2O_2 \rightarrow \cdot OH + Fe^{+++} + OH^-$). Recent studies on the effects of various protective substances have given some support in favor of this idea. It has

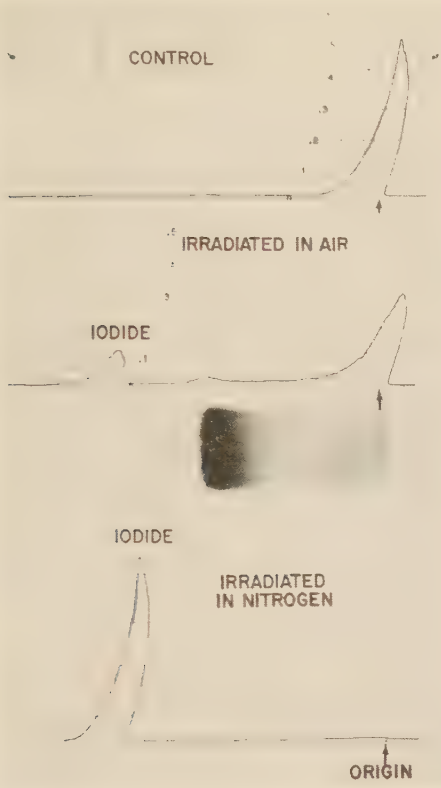

FIGURE 5. Irradiation of insulin-I^{131} in air and in N_2 at identical concentrations of insulin (100 μg./ml.) and doses of irradiation (60 kilorads). In this figure and in FIGURE 1 the paper strips were inadvertently cut short before being photographed.

been observed[5] that the order of protection provided by different analogues within a chemical series is the same for alterations produced by Fenton's reagent or by irradiation. Thus, the relative effectiveness, as protective agents, of individual members of (1) the halide family, (2) the group of sulfur-containing amino acids, and (3) a series of benzene and hydroxybenzene derivatives, follows essentially the same order within each group, as well as among the different groups in both systems. These results also support the current view that protective substances exert their effect through competition for the radicals produced by irradiation of water.

One other chemical change merits some attention. Following irradiation of insulin (either labeled with I^{131} or unlabeled) at concentrations of 200 to 1000 μg./ml. with 100 to 200 kilorad, the solutions reduce ferricyanide with the development of a positive Prussian blue reaction on the subsequent addition of Fe^{+++} ions. Since this reaction is absent (or markedly delayed) in the presence of *p*-chloromercuribenzoate, the reducing effect is tentatively ascribed to the presence of sulfhydryl (SH) groups. A positive *N*-ethyl-

maleimide reaction on paper has also been obtained on several occasions, but the number of SH groups judged to be present (from the extent of the Prussian blue reaction) indicates that the reaction is at the lowest threshold of sensitivity. Native unirradiated insulin that contains no free SH groups

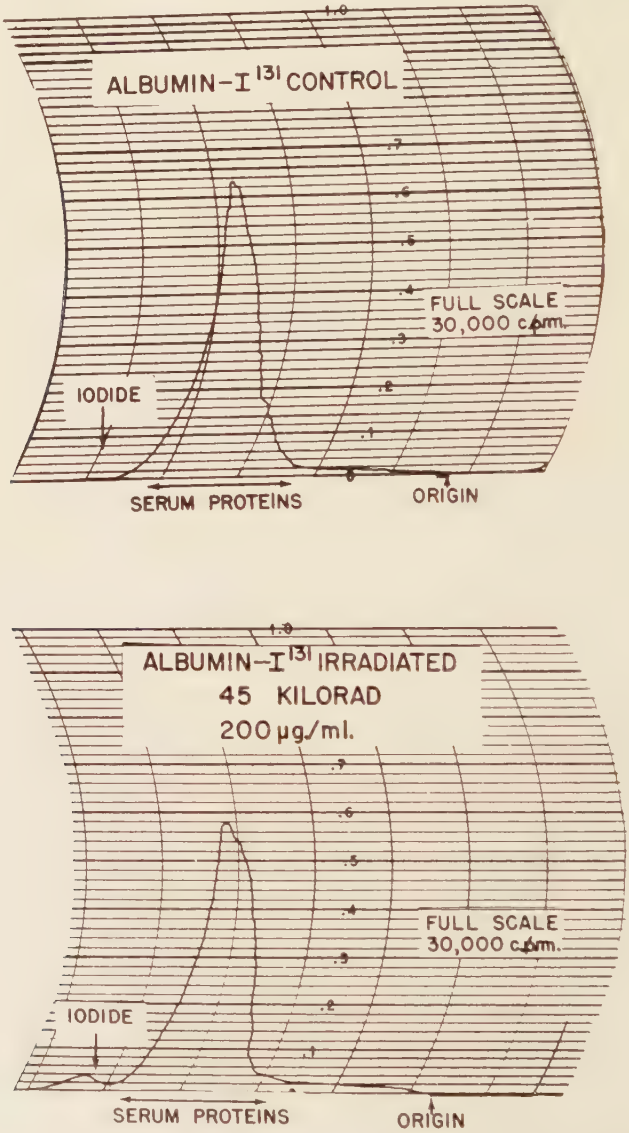

FIGURE 6. Radioactivities of paper strips following electrophoresis for 1½ hr. combined with hydrodynamic flow. Origin indicates site of application of solutions. Phosphate buffer, ionic strength 0.1; constant voltage, 250 V; Whatman 3 MM paper; migration to the left. Reproduced by permission from *The Journal of Clinical Investigation*.[5]

serves as a control and gives negative reactions in both tests. Since serum albumin contains something of the order of less than 1 reactive SH group per molecule, the native species does not give a quickly positive reaction in fresh solution at concentrations of 200 to 10,000 μg./ml. When irradiated with 100 to 200 kilorad, the same solutions give an early positive Prussian blue reaction in the absence of *p* chloromercuribenzoate, but not in its presence.[6] Irradiation in a nitrogen atmosphere enhances these reactions.

In the case of I^{131}-labeled serum albumin, there should be, as a rule, few problems concerned with irradiation damage since, at least for metabolic studies, extremely high specific activity is not generally required. It has been observed that an irradiation dose of 45 kilorad delivered to solutions containing 200 μg./ml. produces no alteration of electrophoretic mobility in paper and a release of only about 3 per cent of the radioactivity as iodide[131] (FIGURE 6).[6] However, the body readily distinguishes approximately half of these preparations as drastically altered material and degrades this fraction in a day or two[6, 12] (FIGURES 7 and 8). The remainder is metabolized at a much slower rate, but still more rapidly than the control albumin-I^{131} that has received 3 to 4 kilorad during its preparation (FIGURE 8). In a concentration of 5 mg./ml., alteration of serum albumin by 45 kilorad was suggestive, but not striking. In one experiment, albumin at a concentration of

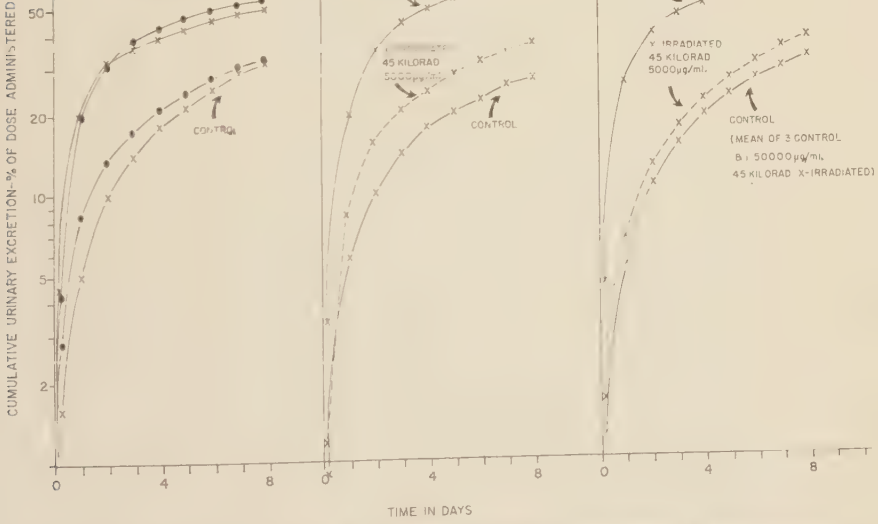

FIGURE 7. Cumulative urinary excretion following intravenous administration of control and X-irradiated albumin-I^{131} solutions to normal human subjects. In the third frame, the curve obtained from the three subjects receiving control albumin-I^{131} solutions and the single subject who had received the albumin-I^{131} irradiated at a concentration of 50 mg./ml. were virtually indistinguishable. Thyroidal uptake was blocked by the administration of Lugol's solution. Reproduced by permission from *The Journal of Clinical Investigation.*[6]

50 mg./ml. was not detectably damaged by this dose of radiation (FIGURE 6). The important point that emerges is that the physicochemical systems may not readily detect alterations that may be discriminated very sensitively by a particular biological system. Thus, an apparent identity with native proteins *in vitro* is an inadequate criterion by which to predict identity *in vivo*. With this in mind, we must also question whether insulin-I^{131} that appears undamaged on electrophoresis is, in fact, unaltered insulin. The only answer that appears justified is the following– that which appears to be altered on electrophoresis is altered; the remainder may or may not be.

More marked electrophoretic alterations of albumin-I^{131} were observed when solutions of lower concentration (20 μg./ml.) were irradiated. Release of iodide^{131} was more prominent, and anomalous migration of other labeled products appeared (FIGURE 9). There was fairly uniform trailing of radioactivity along the entire paper strip that was unrelated to the serum proteins. This appeared to be the result of binding to the paper in which native albumin was not competitive. It may be of some interest to know that the yield of iodide^{131} at similar concentrations (by weight) and irradiation dose levels was much greater for insulin-I^{131} than for albumin-I^{131}. It is not clear whether the tyrosyl residues of albumin are less easily accessible to irradiation-produced radicals or whether other groups are responsible for more effective removal of these radicals.

From the results of these studies, it appears that radiation effects should be of little importance in dealing with labeled proteins of low specific activity.

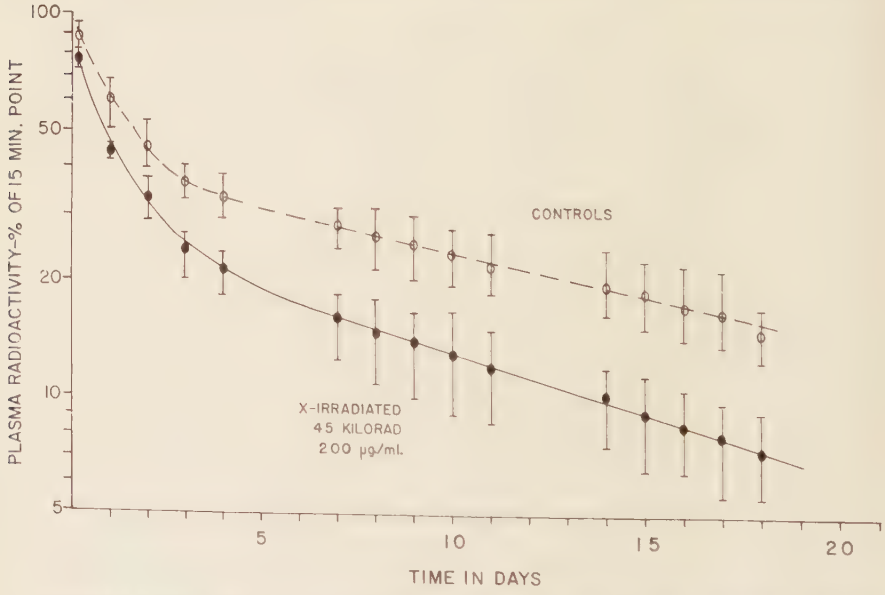

FIGURE 8. Mean plasma concentrations of radioactivities in six subjects receiving control albumin-I^{131} and four subjects receiving irradiated albumin-I^{131}. Horizontal bars indicate extreme range of values for each point. Reproduced by permission from *The Journal of Clinical Investigation*.[6]

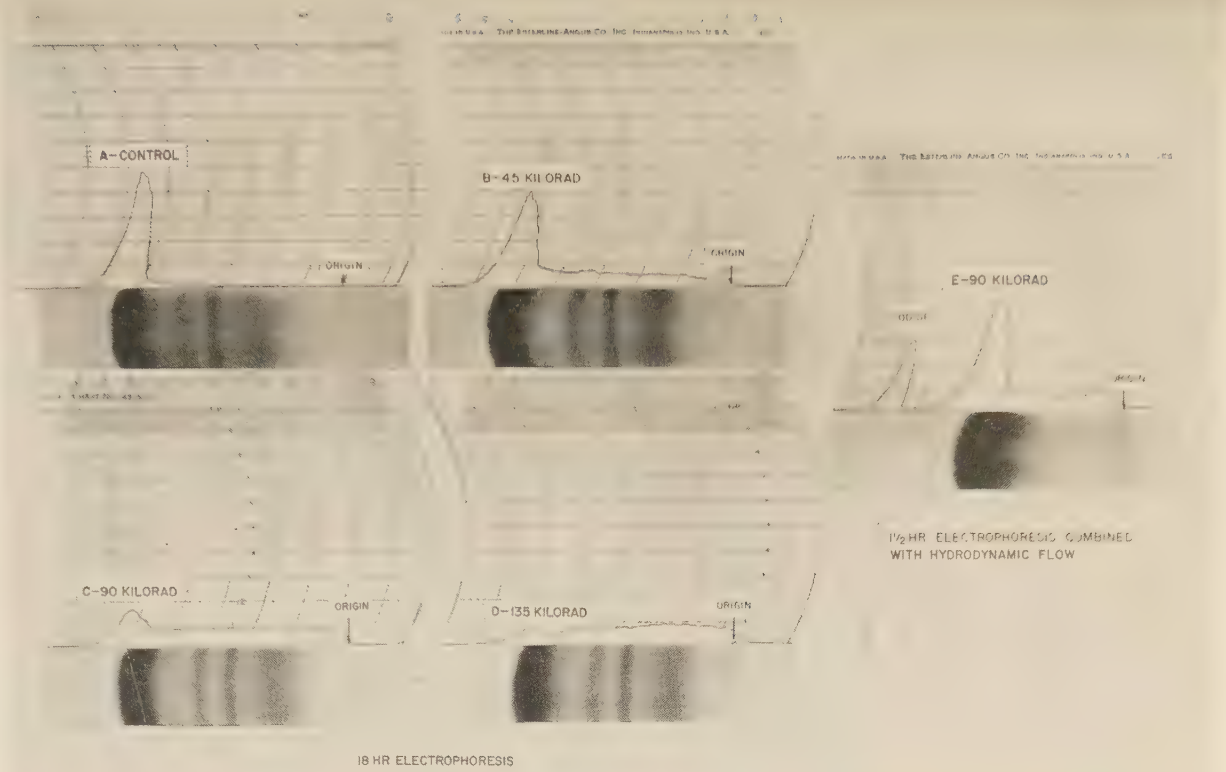

FIGURE 9. Paper-strip radioelectrophoretograms of albumin-I^{131} solutions in plasma. Albumin-I^{131} solutions were subjected to doses of irradiation as indicated at concentrations of albumin of 20 μg./ml. Full-scale reading of 30,000 c.p.m. on all strips. Reproduced by permission from *The Journal of Clinical Investigation*.[5]

Therefore, when the biological nature of the experiment does not demand a preparation of high specific activity, as is most frequently the case in metabolic studies employing albumin I^{131}, such preparations should be avoided. In instances where high specific activity is required, as is frequently the case in studies dealing with such factors as the turnover of tagged hormones and antigen-antibody reactions, attention should be directed toward protection against damage attributable to radiation.

Among the various agents studied, serum albumin is a readily available substance that, in high concentrations, does not injure insulin and is completely protective against any dose of irradiation likely to be absorbed by solutions of I^{131}-labeled proteins. It has, therefore, been our practice to add serum albumin in concentrations of 50 to 100 mg./ml. to all I^{131}-labeled protein preparations immediately following removal of nonprotein-bound I^{131} in order to minimize further damage from irradiation. The high irradiation dose rate delivered during the preparation of solutions of high specific activity, however, requires that the labeling procedure be as short as possible, consistent with adequate yields, and that nonprotein-bound I^{131} be removed as quickly as possible.

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Part II. Applications

THE RELATIONSHIP OF THE RATES OF SERUM PROTEIN METABOLISM, HETEROLOGOUS SERUM PROTEIN CATABOLISM, AND THE TIME AND MAGNITUDE OF THE ANTIBODY RESPONSE*†

By Frank J. Dixon and William O. Weigle

University of Pittsburgh School of Medicine, Pittsburgh, Pa.

In this study, the catabolism of I^{131}-labeled homologous serum proteins and a variety of heterologous serum proteins was observed in several animal species. This was done in order to discover whether there was any relationship between the rates of catabolism of homologous and heterologous proteins. In addition, the pattern of the immune response to the heterologous proteins was also determined and correlated with the rate of catabolism of antigen.

TABLE 1 shows the half lives of the rates of catabolism of homologous proteins and of nonimmune catabolism of heterologous proteins in the rabbit, guinea pig, rat, and mouse. The rates of nonimmune catabolism of heterologous proteins represent the period of exponential elimination prior to the accelerated terminal elimination that accompanies the formation of antibody. From these data, it is apparent that (1) from species to species there is no constant relationship between the rates at which homologous albumins and globulins are catabolized or the rates at which homologous and heterologous globulins are catabolized; and (2) there is no consistent relationship between the rates at which various heterologous proteins are catabolized in a given species. The rate of elimination of a given protein from the circulation and its subsequent catabolism in a certain species is probably dependent upon the metabolic rate of the host and the "foreignness" of the protein; that is, its inability to stay in the circulation and its resistance to degradation by the enzymes of the host. In this latter regard, it appears that rabbit γ-globulin is unique in its ability to persist in the circulation and to avoid or resist degradation in foreign hosts. It is degraded even more slowly than is homologous globulin in the rat and mouse, and at about the same rate as is homologous globulin in the guinea pig. While it has been noted that proteins fractionated by ammonium sulfate appear to have a longer life in the rabbit than do those fractionated by alcohol, this difference does not hold in the guinea pig.

We next studied the immune response of rabbits and guinea pigs to the various heterologous serum proteins. The first evidence of an antibody

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TABLE 1

HALF LIVES OF HOMOLOGOUS AND HETEROLOGOUS I^{131}-LABELED SERUM PROTEINS

Species	Protein*	Method of fractionation	Half life in days
Rabbit	RGG	Ethanol	4.6 ± 0.8
	RGG	Ammonium sulfate	5.7 ± 1.2
	RSA	Ethanol	5.7 ± 0.3
	BGG	Ethanol	2.2 ± 0.3
	HGG	Ethanol	3.0 ± 0.4
	GPG	Ethanol	1.6 ± 0.3
	HSA	Ethanol	4.1 ± 0.4
	BSA	Ethanol	4.3 ± 0.6
Guinea pig	GPG	Ethanol	5.4 ± 0.3
	GPG	Ammonium sulfate	5.4 ± 0.9
	GPSA	Ethanol	2.8 ± 0.2
	GPSA	Ammonium sulfate	2.6 ± 0.2
	RGG	Ethanol	5.0 ± 0.3
	RGG[1]	Ammonium sulfate	4.6
	BGG	Ammonium sulfate	1.8 ± 0.1
	HGG	Ammonium sulfate	1.9 ± 0.2
	RSA	Ammonium sulfate	2.3 ± 0.1
	BSA	Ammonium sulfate	2.3 ± 0.1
Rat	Rat G	Ammonium sulfate	2.6 ± 0.2
	Rat SA	Ammonium sulfate	2.1
	RGG	Ethanol	6.7 ± 0.7
	BGG	Ethanol	3.0
	RSA	Ethanol	1.1 ± 0.1
	BSA	Ethanol	1.3 ± 0.1
Mouse	MGG	Ammonium sulfate	1.9
	MSA	Electrophoresis	1.2
	RGG	Ammonium sulfate	5.1
	BGG	Ethanol	1.5
	HSA[2]	Ethanol	1.5

*Symbols: RGG = rabbit γ-globulin; BGG = bovine γ-globulin; HGG = human γ-globulin; MGG = mouse γ-globulin; GPG = guinea pig globulin; Rat G = rat globulin; RSA = rabbit serum albumin; HSA = human serum albumin; BSA = bovine serum albumin; GPSA = guinea pig serum albumin; MSA = mouse serum albumin; Rat SA = rat serum albumin.

response to these proteins is an increased rate of their elimination from the circulation. This rapid elimination is caused by the combination of antibody with antigen and the removal of the complexes from the blood. In both the rabbit and the guinea pig, antibody-antigen complexes can be demonstrated in the circulation during this phase of rapid elimination. The duration of the period of induction prior to the synthesis of antibody or to the rapid elimination of antigen and the amount of precipitating antibody formed were compared with the rate of nonimmune catabolism of the protein to see whether these processes were related in any way. From the observations in TABLE 2 it appears that (1) while there is some relationship between rate of catabolism and length of induction period for antigens in a single species, there is no such relationship from species to species; (2) the antibody response of the rabbit is more prompt than that of the guinea pig; and (3)

TABLE 2
CORRELATION OF ANTIGEN CATABOLISM AND ANTIBODY RESPONSE

Species	Protein*	Nonimmune catabolism 1/2 life	Induction period—days	Antibody response
Rabbit.....	BGG	2.2	5	+++
	HGG	3.0	5	++
	BSA	4.3	7–9	+++
	HSA	4.1	7–9	++
Guinea pig.....	BGG	1.8	7–9	+++
	HGG	1.9	8–10	±
	RGG	5.0	10–13	++
	BSA	2.3	—	0
	RSA	2.3	9–11	0

*Symbols: BGG = bovine γ-globulin; HGG = human γ-globulin; BSA = bovine serum albumin; HSA = human serum albumin; RGG = rabbit γ-globulin; RSA = rabbit serum albumin.

there is little or no relationship between the size of the antibody response and the rate of antigen catabolism or the induction period.

It is likely that the size of the antibody response is related to particular antigenic characteristics of the heterologous protein. These characteristics are separate from those that determine the rate of nonimmune elimination from the circulation and nonimmune catabolism of the protein. These separate characteristics of the proteins might be considered immunological on the one hand and physiological on the other. It is of interest to know that there is little or no relationship between the phylogenetic proximity of the host and the source of antigen and either the rate of nonimmune catabolism or size of antibody response.

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CURRENT STATUS OF THE TISSUE LOCALIZATION OF I^{131}-LABELED ANTITISSUE ANTIBODIES*

By David Pressman

Department of Biochemistry Research, Roswell Park Memorial Institute and the Roswell Park Division of the University of Buffalo Graduate School of Arts and Sciences, Buffalo, N. Y.

When an animal is injected with homogenized tissue from another species, antibodies are formed against the various components of the injected material. Some of these antibodies may have the property of being able to localize in the particular tissue against which the antibody was formed when injected into the species furnishing the tissue. Other antibodies may react *in vitro* with components of the tissue without being able to localize in the tissue. The localizing properties[1] are most easily followed by iodinating the antibody with radioactive iodine I^{131} and following the localization of the radioactivity.†

The process of determining the localizing properties of an antiserum consists of fractionating the antiserum to yield the globulin fraction that contains the antibody; labeling this with radioactive iodine by an iodination procedure; injecting the radioiodinated globulin intravenously; and subsequently perfusing the animal to remove circulating radioactivity and assaying various parts of the animal thus injected for localization of radioactivity.

It has been shown many times already that antibody directed against an antigen is not destroyed by moderate iodination, although extensive iodination does destroy antibody activity.[7, 8] Indeed, it has been shown that the antibody can be highly iodinated without destroying its ability to react with antigen if the specific site is protected against alteration during iodination by combination with the original hapten or antigen against which it was directed.[9, 10] The iodine label in trace-labeled antibody has been shown, by Melcher and Masouredis,[11] to be stable *in vivo* in guinea pigs and does not have a metabolic fate independent of the antibody molecule itself.

Systems Studied

Localizing antibodies have been shown, by radioactive means, to exist in antisera prepared against tissues from rats and mice, as shown in TABLE 1. Both normal and tumor tissues have been used. Localizing antibodies studied by radio label methods have been produced in rabbits and ducks.[30] In all the cases mentioned, antibodies were formed that would localize in the particu

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† Radioactive I^{124}, I^{131}, I^{133}, S^{35}, and C^{14} have also been used as radioactive tags on localizing antibody.[2-4] Fluorescein-labeled antibody capable of reacting with the injected antibody has also been used for showing the localizing properties of an antitissue antibody.[5, 6]

Table 1

Tissues Against Which I^{131}-Localizing Antibodies Have Been Prepared

Rat kidney[1,2,12–16]	Mouse kidney[27,28]
Rat liver[17,18]	Mouse Wagner osteogenic sarcoma[29]
Rat lung[19,20]	
Rat placenta[21]	
Rat aorta[22]	
Rat lymph node[12,23]	
Rat spleen[24]	
Rat pancreas[18]	
Rat adrenal[13]	
Rat ovary[12]	
Rat Murphy lymphosarcoma[12,25]	
Rat Walker carcinoma[14]	
Rat Flexner-Jobling carcinoma[26]	

lar tissue against which the antibodies were formed*. However, extensive cross reactions were observed, since antibodies would localize in other tissues as well.

Cross reactions are due to antibody formed against common components present in the various tissues. They are not caused by antibodies directed against blood constituents, since antisera to mouse plasma[28] or red cells[32] show no localization different from that of normal sera.

In order to overcome the cross reactions, methods have been developed for specifically purifying antibody. Nevertheless, it is frequently possible to demonstrate, without any specific purification procedure, that antibodies specific for a particular tissue are present even when an extensive amount of cross-reacting antibodies are also present. This is especially true when antibodies prepared against a tissue localize in that particular tissue, whereas antibodies prepared against other tissue show little or no localization in the particular tissue of interest. This is the case with antirat-lung sera[19,20] or antirat-Murphy-lymphosarcoma sera.[23,25] Antiserum prepared against rat lung shows localization in rat-lung tissue as well as a high localization in kidney and other tissues. However, antikidney serum shows little localization in lung tissue. The presence of specific lung-localizing antibodies is thus demonstrated. Similarly, in the anti-Murphy-lymphosarcoma system, antibodies prepared against the tumor show tumor and kidney localizing properties, whereas the antibodies to kidney show no localization in tumor.

Other evidence for differences between organs with respect to localization of antibodies lies in the fact that certain antibodies in a given serum localize preferentially in a particular organ, while others localize preferentially in another organ, as is evident by determining the localizing properties of 2 sera prepared against the same or different tissues. The localization

* Some tissues have been investigated, and no localizing antibodies were found in them. For example, Mason *et al.*[31] found that antibodies formed against granules of melanin of the Harding-Passy melanoma did not localize in the melanoma. Wissler *et al.*[26] found no tumor-localizing antibodies in antibodies against the Jensen sarcoma of 26 rats. These are examples of nonlocalizing antibody, which will be discussed below.

patterns are different, sometimes markedly so, even for 2 antisera prepared against the same tissue. The differences between the sera must be due to the individual response of the animals producing the sera to the mixture of antigens injected. The fact that the patterns of localization are different is proof that antibodies of different specificities are formed, and that certain of these localize preferentially in a particular tissue. Further evidence for heterogeneity and specificity is found with antikidney serum. Antiserum produced against rat kidney contains antibodies cross-reacting with liver. However, a large portion of the kidney-localizing activity is not cross reactive, and this is shown by passive transfer through nephrectomized and normal rats. Both transfers remove liver-localizing activity almost completely in 30 minutes. However, the nephrectomized rat removes only 40 per cent of the kidney-localizing activity, while the normal animal removes 75 per cent, which indicates that about 35 per cent of the kidney-localizing activity is not capable of localizing elsewhere than in the kidney in the intact animal.[33] This finding is in accord with the results of Sarre and Wirtz,[34] who obtained evidence for antibodies of only kidney-localizing activity on the basis of studies of nephrotoxicity.[34]

The Specific Purification of Localizing Antibody

Specific purification can be achieved either by *in vivo* methods or by *in vitro* methods. In the *in vivo* methods,[16, 17, 35] labeled localizing antibody is injected into an animal, and time is permitted for localization to take place. Subsequently the animal is perfused with saline to remove circulating radioactivity, the tissue is homogenized, and the antibody is eluted from the tissue by alkali or heat and reinjected into recipient animals. There result a marked purification and specificity of antibody. Such purification procedures have been carried out with antiserum to rat kidney, lung, and liver.

In the *in vitro* methods, the antibody is absorbed on the insoluble sediment* of the tissue of interest.[13, 14, 23, 24, 25] The sediment is then washed, and the antibody is eluted by either heat or alkaline treatment. The eluted antibody then has the property of localizing to a higher degree than previously in the tissue used for the specific absorption. The antibody can be purified further by a second absorption and elution procedure. Cross-reacting antibodies can be further removed by absorbing them from the solution with a heterologous tissue. An alternative procedure that can be performed to advantage simultaneously is to absorb out cross-reacting antibodies prior to the concentrating absorption and elution procedure. This is done by absorbing the antiserum with a sediment of a cross-reacting tissue and subsequently absorbing the remaining antibody onto a sediment of the tissue of choice. These methods result in increased specificity in the product and have been used to purify various tissue localizing antibodies.

The sediments of tissues are used because the antigen responsible for the formation and localization of localizing antibodies is associated, at least in part, with the insoluble portions of the tissue. This has been clearly shown

* The insoluble sediment is obtained from the homogenate of a tissue by repeated washing of the material sedimented at low centrifugal force.

by the fact that the saline-washed sediment of a tissue effectively removes localizing antibody from solution.[36] Moreover, the soluble components of kidney tissue are not effective in neutralizing kidney-localizing antibody.

One problem in connection with production of antibodies against tissues or organs is the fact that the tissues and organs are complex mixtures of various types of cells and other structures, with the result that it is not possible to achieve very extensive purification by using such mixtures. This problem has been attacked by the partial separation of various structures of a tissue.[24] Liver, lung, spleen, and kidney tissues were fractionated crudely into two portions by pushing the organ through a tissue press. The material left behind in the press was composed primarily of the large blood vessel connective tissue, whereas the cellular material and capillaries passed through the mesh. Sediments prepared from such fractionation showed very interesting properties. The large blood-vessel tissue and connective tissue, when used for purification of antibodies from antikidney, antiliver, antilung, and antispleen sera, resulted in the isolation of the more cross-reacting antibodies, while the fractions obtained by using the sediment from the cellular fraction showed the greater specificity. These results would indicate that the cross-reacting antigens are associated with connective tissue and large-blood-vessel components. This is especially true for the case of the kidney antigens responsible for localization in kidney tissue.

The problem of cross-reacting antibodies has been largely overcome, in the case of antibodies prepared against the Murphy lymphosarcoma, by using the ascites tumor form for the production of antibodies and for specific purification.[25] The ascites tumor cells are free of the connective tissue components present in solid tumors or normal tissues and therefore yield preparations showing less cross reaction when assayed in animals bearing the solid form of the tumor. Antisera prepared against the solid form contain appreciable amounts of cross-reacting antibody.

Isolation of Antigenic Components Responsible for Localization

In the case of kidney tissue, it has been possible to obtain soluble materials that will neutralize kidney-localizing activity.[37] This has been done by digesting kidney tissue with trypsin; the products thus obtained are able to neutralize kidney-localizing activity. That such products are capable of neutralizing nephrotoxic antibody has already been shown by Cole, Cromartie, and Watson[38] and by Goodman and Baxter.[39] The trypsin-digested material[37] was further fractionated by alcohol precipitation and was digested with ribonuclease and desoxyribonuclease to such an extent that the products contained neither phosphorus by chemical test nor nucleic acid components as determined spectrophotometrically. The material was still able to neutralize kidney-localizing activity. However, the soluble substances either before or after the treatment with nuclease were unable to neutralize kidney-localizing activity completely. This would suggest that there are localizing antibodies directed against more than one kidney antigen and that only some of them are neutralized by the soluble substances isolated.

Localization of Antibody in Heterologous Species

We have shown that antisera prepared in rabbits against mouse kidney localizes in rat kidney.[28] Spar, Bale, Wolfe, and Goodland[40] have presented evidence that, when rabbits produce antibody to rat kidney, some of this antibody is retained by cross reaction in the rabbit kidney.

Mechanism of Localization

Localization takes place when the antibody comes in contact with the antigen upon which it can localize. Whether the antibody is localizing or nonlocalizing depends entirely on whether the antibody can come in rapid and intimate contact either with the antigen against which it was formed or with a cross-reacting antigen.

Antigenic substances are present in various parts of the tissue. If they are in immediate contact with the blood (that is, in the vascular bed), localization can take place as the blood passes this structure. Localization will then be a function of the rate of flow of blood past the structure. Antibody that is not localized will be recirculated through the blood. If the antigenic structure responsible for localization is present on the surface of the cell in contact with extravascular fluids, but not in immediate contact with the blood, the rate of localization will depend on the rate of passage of extravascular fluid past the structure. The rate of passage of extravascular fluid past a particular structure is much slower than the rate of passage of blood, and the time required for passage of large fractions of the injected antibody past the structure would be greatly increased. If the antibody is formed against a structure inside the cell, there is not much chance for localization of an appreciable amount of such antibody to take place. If localization is to take place inside the cell, it would be necessary for the specific antibody to enter the particular cells to react with the antigen. Similar antibodies also would be able to enter cells of other tissue, since entrance to cells would necessarily be nonspecific (if entrance were specific, the selection and, consequently, the localization would be determined at the cell surface, as in the case already mentioned). If metabolized in the heterologous tissue, these potentially localizing antibodies would be lost for the localization process in the cells containing the specific antigen. As a result, there would be a more reduced localization than would be possible on the cells.

The fraction of the amount of antibody injected that can localize in a particular organ will show decrease for the three possible methods of localization in the following order: (1) localization directly from blood stream; (2) localization from extravascular fluid on surfaces of cells; and (3) localization inside cells, since the metabolism during the increased time required for localization required in the latter processes would very effectively decrease the amount of antibody available for localization.

The evidence that we have obtained thus far indicates that the localization takes place in the vascular bed or directly from the blood stream.[41] Investigation of the rate of localization of antirat-kidney antibodies indicates that

the kidney-localizing component in the antiserum disappears very rapidly.[33] There appear to be 2 kidney-localizing components: the first with a half time of 4 min., and the other with a half time of 80 min. The half life in the blood stream for the more rapidly localizing component is that expected for complete removal of antibody as it passes through the kidney, calculated on the basis of published values for blood flow through that organ. The 80-min. half time is appreciably slower, but probably it is also due to localization in the vascular bed. Liver-localizing activity also shows a similar 2-component pattern with half times of 2 min. and 30 min., respectively.

If such rapid localization is to occur, it must take place on a structure that is in very close contact with the circulation. The presence of antibodies with two different half lives localizing in a particular organ is further evidence of the fact that more than one localizing antibody can be formed against any particular structure.

Nonspecific Uptake of Radioactivity

In all of these studies of localization, there was a comparison made with the uptake by the tissues of a similarly labeled control preparation, usually the corresponding fraction of normal serum or an antiserum of a different specificity. In the case of some tissues, those of tumors, for example, the nonspecific localization may be especially important since tumor tissue is known to pick up foreign proteins nonspecifically. Specific localization is the difference between the localization from the antitissue antibody preparation and the control preparations. Frequently, the purification procedures described above also result in the separation of material showing increased localization from control sera.[35, 42] This is apparently due to the isolation of certain minor components of control sera capable of reacting most strongly with these preparations. The total amount of localizing material so isolated from any control serum has always been low as compared to the amount isolated from an antiserum.

Paired Labels

In order to be able to control experiments more effectively where it is desirable or essential to determine control localization simultaneously with antiserum localization (as, for example, may be the case for studies with human tumors where only a particular tumor patient is available for a single experiment), we have developed a paired-label technique in which antibody is labeled with 1 isotope and control material is labeled with another, so that both substances may be injected simultaneously and their relative localization properties determined in the same individual. We have been able to develop such paired labels, using I^{131} as one label and I^{133} or I^{124} as the other label.[3, 4] For use with experiments that require a longer half life of isotopes than those of I^{133} or I^{124}, still another label was developed, namely, the *p*-iodobenzoyl label.[4] In this case, the protein is reacted with *p*-iodobenzoyl chloride; either the carbon or the iodine is labeled. Both of the paired labels are comparable. The advantage of using a paired label in which exactly the same chemical reaction has been used in the labeling process and in which

the same chemical alteration of the protein has taken place is self-evident. Previous experiments using S^{35} azobenzene sulfonic acid groups and I^{131} indicated that the different labels affected the nonspecific localization differently.[2]

Duration of Fixation of Localized Antibody

Antibody that is localized in tissue remains in the tissue for different lengths of time. Antikidney antibody localized in mice has a half life of 20 days in the kidney and also in the liver, although there is a preliminary shorter half life component in the liver of approximately 3 days.[28] In the case of rats injected with antiserum prepared against rat lung, the observed half life of antibody was 25 days in the kidney, 12 days in the liver, and 6½ days in the lung.[20] The very long half life of the localized antibody in a particular tissue indicates that the antibody is localized in such a way that it is not easily metabolized once it has localized.

The Precise Localization of Kidney-Localizing Antibodies

The localization in the kidney has been found to take place to a high concentration in the glomerular tuft of the kidney. This is shown clearly by radioautographs obtained from sections of kidney tissue from animals receiving radiolabeled antikidney serum as compared with those from animals receiving radiolabeled control serum.[2, 27] A similar demonstration has been made using fluorescein-labeled antirabbit globulin serum prepared in chickens to demonstrate the localization of the rabbit antikidney antibodies.[5, 6]

Nonlocalizing Antitissue Antibodies

The antibody prepared against a tissue homogenate contains both localizing and nonlocalizing antibodies, and this can be demonstrated easily in various experiments. Such antisera will give a precipitate with a soluble component of a tissue homogenate, but the formation of this precipitate and its removal do not remove kidney-localizing antibodies.[36] Thus, we have evidence that there are antibodies formed against soluble components that are not the localizing antibodies. These antibodies can react with the soluble components of the kidney tissue and are not localizing because they cannot reach the antigen to be localized. However, if they are brought in contact with the antigen by some *in vitro* process, such as breaking up the cells or, perhaps, slicing the cells and exposing them to antibody so that contact is made with the particular antigen, combination can take place. Thus, Hill and Cruickshank[43] showed that antikidney antibody will stain sections of kidney tissue in regions other than the glomerular tuft. On this basis, these investigators claim that the fluorescein-label technique shows localizations other than those observed with radioiodinated preparations. However, there is a difference in administration; that is, in one case the antibody is administered intravenously and there is a dependence on an *in vivo* localization, whereas in the experiments reported by Hill and Cruickshank the localization was really *in vitro*, where the antigen and antibody are brought together artificially.

Clayton[44] has used radioiodinated antisera as a histological stain and followed staining by radioautography.

Cytotoxic Properties of Localizing Antibodies

It is practically impossible to prove that all localizing antibodies are cytotoxic. However, it is apparent that antibodies, in order to be cytotoxic, probably must localize in the tissue affected. There are, indeed, strong parallels between the properties of cytotoxic and localizing antibodies in several systems. This is especially true in the case of antikidney antibodies. The nephrotoxic antikidney antibodies and the localizing antikidney antibodies show very parallel properties. Both are absorbed by kidney homogenate, and both are neutralized (localizing in part) by trypsin digest of the kidney tissue.

The localization of radioiodinated antibody is not due to the effect of an antibody's exhibiting a cytotoxic effect on the tissue so that the tissue subsequently picks up iodinated protein nonspecifically. This has been clearly demonstrated, since the injection of antirat-kidney antibody along with radioiodinated antiovalbumin does not result in an uptake of radioactivity.[15]

Cytotoxic Properties of Radioiodinated Antisera Due to Radioactivity

In the administration of radioactive antitissue antibody there is the possibility of developing a therapy against cancer by localizing a physiologically active amount of radioactivity in the tumor. It has been pointed out that it should be possible to localize very large quantities of radioactivity in a tissue if the localization is similar to that which takes place in the kidney. It is known that a large amount of antibody can be localized in that organ (0.5 mg. gm. of kidney).[45] If the amount of iodine localized (2γ/gm.) were entirely carrier-free I^{131}, a concentration of 250 mc. of iodine per gm. of tissue could be achieved, and this would certainly have a cytotoxic effect due to radiation.

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THE PRIMARY EQUILIBRIUM BETWEEN ANTIGEN AND ANTIBODY*

By David W. Talmage

Department of Medicine, University of Chicago, Chicago, Ill.

When antigen (*Ag*) and antibody (*Ab*) are mixed in solution, there occurs a reaction that is based on an affinity between the reactive sites on the molecules of each. A large body of evidence now exists to indicate that this reaction is reversible and follows the laws of mass action.[1] Thus,

$$Ag + Ab \rightleftarrows AgAb \qquad (1)$$

Such secondary manifestations of the antigen-antibody reaction as precipitation, agglutination, and hemolysis are in reality second-stage reactions involving 2 or more antigen-antibody complexes. An understanding of the mechanism of these second-stage reactions requires, first of all, a knowledge of the various factors that influence the primary reaction between antigen and antibody.

Numerous methods have been developed for measuring the constants of the primary equilibrium between antigen and antibody. These methods all involve the measurement of 1 of the 2 components (antigen or antibody) in both its free and bound states. Probably the earliest method used for this purpose was that of toxin neutralization.[2-4] In this procedure a known amount of toxin is mixed with antibody, and sufficient time is allowed for the reaction to reach equilibrium. The amount of free toxin is then measured by biological assay. Experimental results obtained with this technique have led to the demonstration of the diversity of antibody with respect to both avidity[3, 4] and biological activity.[5, 6] These studies also have led to the development of the concepts of antibody production advanced by Jerne in his "Natural Selection Theory."[7]

Another method used for measuring the primary antigen-antibody reaction is equilibrium dialysis.[8] In this case, the concentration of free dialyzable hapten is measured on the outside of a dialysis membrane by the use of a dye or radioactive label. The measurement of total concentration of hapten on the inside or reaction side of the membrane permits the determination of bound hapten by subtraction. Since the first two methods are limited to toxic antigens or to haptens, the search for additional techniques continues. Free antigen is separable from bound antigen by virtue of their different electrophoretic[9] and centrifugal[10] rates of migration. Bound antigen may be separated from free antigen by any method of selectively precipitating the serum globulins as, for example, with ammonium sulfate[11] or an antiglobulin serum.[12] Free antibody may be separated from bound antibody

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by making use of the many naturally occurring insoluble antigens.[1, 13, 14] In all of these techniques, it is essential that the conditions of equilibrium do not permit a significant degree of second-stage reaction which, by removing the end product of the primary reaction, may influence the primary equilibrium. Another requisite is that the process of separation should not influence the equilibrium. The importance of radioisotopes to these techniques lies in the fact that they permit the easy quantitation of antigen or antibody after the separation of their free and bound states has been accomplished.

The primary equilibrium between antigen and antibody is best described in terms of the dissociation constant K_D, which is equal to the product of the concentrations of free antigen and antibody divided by the concentration of the antigen-antibody complex:

$$K_D = \frac{(Ag)(Ab)}{AgAb} \tag{2}$$

For biological studies, the dissociation constant K_D is preferred to its reciprocal[1, 8, 10] because K_D, like the Michaelis-Menten constant of enzyme activity, may be expressed directly in terms of concentration of free antigen. To demonstrate this relationship, the above equation may be rewritten as

$$\frac{AgAb}{Ab} = \frac{Ag}{K_D} \tag{3}$$

The ratio of bound to unbound antibody is equal to the ratio of the concentration of free antigen to K_D. When $Ag = K_D$, then $AgAb = Ab$, and 50 per cent of the antibody is bound. Similarly, when $Ag = 4\ K_D$, the ratio of bound to unbound antibody is 4, and thus 80 per cent of the antibody is bound. A shift in concentration of free antigen from one-fourth K_D to 4 K_D changes the percentage of antibody binding from 20 to 80 per cent. Changes in concentration of antigen below and above these limits have much less striking effects. The dissociation constant represents the center of a narrow zone of concentration of antigen that is very critical to antibody binding. This relationship between the concentration of antigen and K_D should apply also to the cellular fixation of antigen which precedes synthesis of antibody. When the concentration of free antigen is many times the dissociation constant of the equilibrium between antigen and cell receptor, there is saturation of the receptors. Therefore, a further increase in dose of antigen should have relatively slight effect on the antibody response. However, there should be a critical dose of antigen below which the antibody response declines very rapidly. The extracellular concentration established by this critical dose of antigen will approximate the dissociation constant of the antigen-cell receptor equilibrium, and it is therefore a measure of the affinity between the antigen and the cell. A comparison between this affinity and that of the antigen for antibody will permit an experimental test of Paul Ehrlich's long neglected side-chain theory of the formation of antibody.[15] According to this theory, antibodies are cast-off cellular receptors that are stimulated to replicate when they are bound by antigen.

In order to make a comparison between antibody and cell receptor, it is necessary to use a pure, soluble antigen that is freely diffusible, so that the initial serum concentration of antigen is a measure of the maximum concentration of antigen to which the cells are exposed. At the same time, a method must be available for determining the dissociation constant of the antigen-antibody equilibrium. The ammonium sulfate technique of Farr[11] was the first that permitted measurement of the equilibrium between antibody and a soluble nontoxic protein antigen. The method requires a pure I^{131}-labeled soluble protein that is not precipitated by 50 per cent saturated ammonium sulfate; for example, bovine serum albumin (BSA). It was established by Farr that the ammonium sulfate did not affect the equilibrium between BSA and anti-BSA but, rather, froze this equilibrium by precipitating the bound antigen along with the globulins of the serum. The concentration of free and bound antigen then can be obtained by counting the supernatant and precipitate respectively. This gives 2 of the 4 factors (*Ag* and *AgAb*) in the equation

$$K_D = \frac{(Ag)(Ab)}{AgAb} \tag{4}$$

If this determination is made under 2 different experimental conditions of antigen and/or antibody concentration, the 2 unknowns K_D and *Ab* may be solved algebraically. Farr has solved this set of equations for K_D in terms of *S*, the free antigen concentration, and *TP*, the amount of antigen bound by 1 ml. of antiserum, as follows:

$$K = \frac{T_1P_1 - T_2P_2}{\frac{T_2P_2}{S_2} - \frac{T_1P_1}{S_1}} = \frac{\Delta TP}{\Delta \frac{TP}{S}} \tag{5}$$

This equation and the previous ones may be summarized as follows: A relatively avid antigen-antibody reaction has a relatively low dissociation constant and is saturated by a comparably low concentration of free antigen. If this saturating concentration of antigen is below the levels of antigen that are measured experimentally, then relatively little change in the amount of antigen bound (ΔTP) is produced by observed changes in the concentration of free antigen (ΔS). On the other hand, a nonavid antigen-antibody reaction has a high dissociation constant and requires a high concentration of free antigen to maintain saturation of antibody binding sites, and the amount of antigen binding varies greatly with the concentration of free antigen. By plotting antigen binding (*TP*) against the ratio of antigen binding to free antigen concentration (TP/S) one obtains a line the slope of which is equal to K_D. FIGURE 1, plotted in this way, illustrates a number of differences between the antiserum obtained from a rabbit 12 days after the single intravenous injection of 100 mg. of bovine serum albumin and the antiserum obtained from the same rabbit 38 days later. The binding of antigen by the antiserum of day 12 varies greatly with the concentration of free antigen; its plot has a steep slope, and it has a dissociation constant

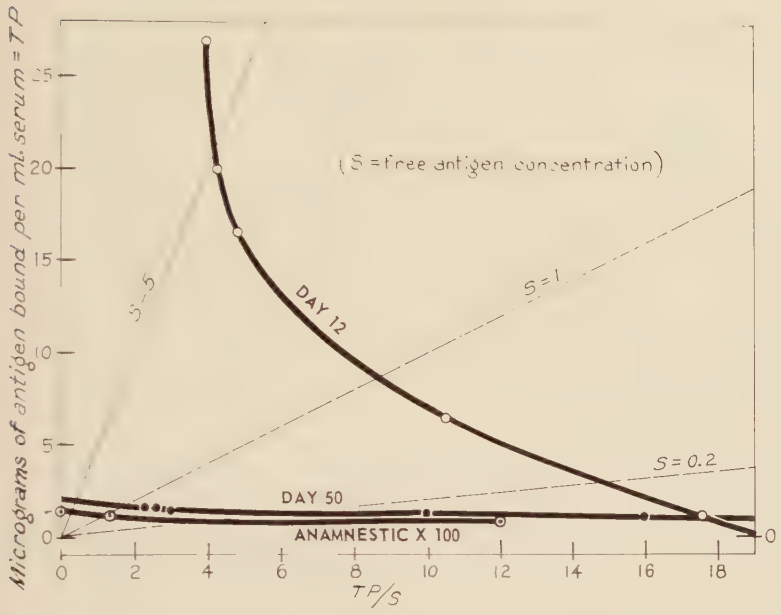

FIGURE 1. The relationship between concentration of free antigen and antigen binding by antisera obtained 12 days and 50 days after a single intravenous injection into a rabbit of 100 mg. of bovine serum albumin and by an antiserum obtained 7 days after a second injection of the same antigen.

of approximately 1 μg. of antigen N. Preliminary experiments indicate that this is close to the critical level of initial serum antigen concentration required to induce a primary antibody response. The antiserum of day 50 and the serum obtained after a second injection of antigen both have dissociation constants of approximately 0.02 μg. This is comparable to the low dosage of antigen required to sensitize an animal and induce the anamnestic response in it. These experiments suggest that antibody responses are stimulated by an equilibrium reaction between antigen and a pre-existing receptor with an affinity for antigen similar to the antibody ultimately formed.[16] If extended and confirmed, these results would represent a striking experimental confirmation of Ehrlich's original concepts of antibody formation. Jerne's "Natural Selection Theory"[7] also postulates that antibody production is the multiplication of selected pre-existing receptors. However, the fact that the change in character of the antibody occurred between day 12 and day 50 in the absence of circulating antigen is not consistent with the details of the mechanism of selection postulated by Jerne.

To this point in this paper, avidity of the antigen-antibody reaction has been considered in terms of the dissociation constant of the primary equilibrium and the concentration of free antigen required to saturate antibody binding. More basic thermodynamic constants, such as free energy of formation (ΔF), enthalpy (ΔH), and entropy (ΔS), have been determined for various antigen-antibody reactions and have demonstrated the diversity

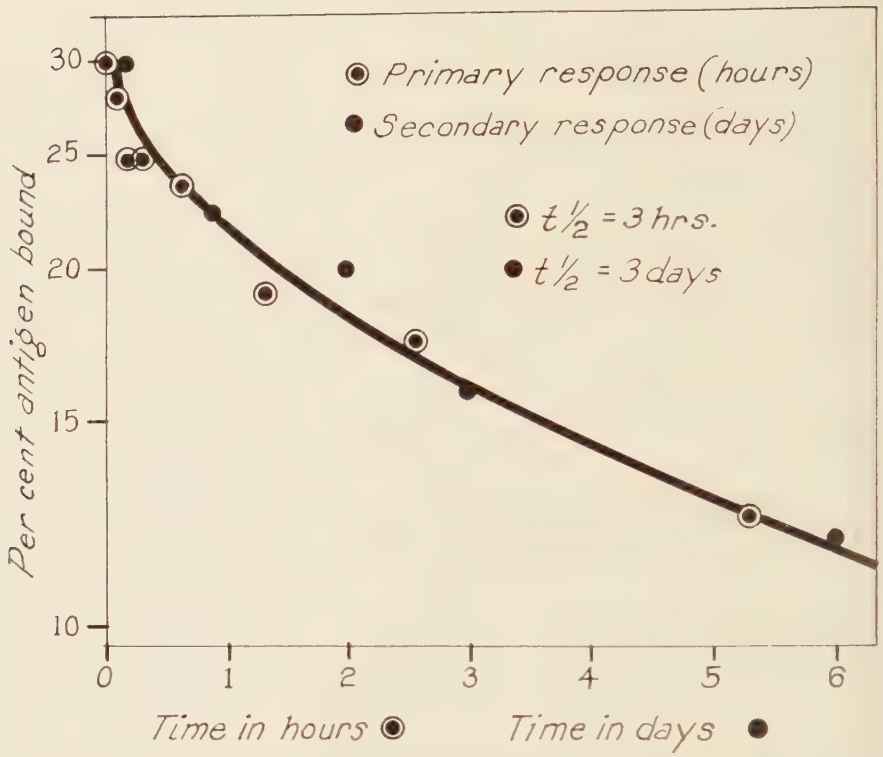

FIGURE 2. The half-binding time for bovine serum albumin of antisera obtained 16 days after the first injection and 7 days after the second injection of 100 mg. of bovine serum albumin.

with which antigens and antibodies are bound together.[1, 10] Probably of more biological significance than these constants is the half-binding time of the antigen-antibody complex. A determination of the half-binding time of the two antisera illustrated in FIGURE 1 may explain the fact that the production of the more avid anti-BSA reaches a peak later than the less avid anti-BSA. The half-binding time for I^{131}-labeled BSA of the first and second response antisera has been obtained by first allowing the antiserum to equilibrate with the labeled antigen and then following the dissociation of these complexes in the presence of a large excess of unlabeled antigen. By saturating all free antibody sites, the unlabeled antigen prevents any significant reassociation of labeled antigen and antibody. FIGURE 2 illustrates the differences between the first and second response antisera. The half-binding time of the first response serum was three hours, that of the second response serum three days. If antibody responses are the result of a reaction between antigen and an antibodylike cell receptor, then the later peak of the more avid antibody may be explained by the longer life of the complex formed between cell receptor and antigen.

The Mechanism of Immune Hemolysis

The results of the reaction between bovine serum albumin and anti-BSA illustrate the importance to the problem of antibody production of a study of the primary antigen-antibody equilibrium. This primary equilibrium plays an equally important role in determining the secondary biological manifestations of the antigen-antibody union. The model used in the study of this latter problem has been the hemolysis of sheep red cells by rabbit antibody and guinea pig complement. The biological end result, hemolysis, can be measured simply and accurately. However, the primary antigen-antibody reaction preceding hemolysis presents certain problems. While it might be possible to dissolve, purify, and label the insoluble antigens of the surface of the red cell, a different process undoubtedly would be required for each of the several antigens involved. One antigen has been shown to be a lipopolysaccharide; others are undoubtedly protein. To avoid performing this formidable task, an alternative approach has been used; namely, that of purifying and labeling the antibodies. In this case, the equilibrium between antigen and antibody may be frozen by centrifuging the antigen and the bound antibody away from the free antibody. This procedure is used, first of all, in separating the labeled antibody from the host of other labeled globulins, so that subsequent determinations of radioactivity may indicate with confidence the amount of antibody present.[13]

The procedure is performed in three steps:

First, the labeled antibody is absorbed by the insoluble antigen using relative amounts of antigen and antibody, which just saturate the antigen. This leaves the bulk of the labeled nonantibody globulin in the supernatant.

Second, any labeled globulin nonspecifically absorbed is washed free with the aid of a carrier globulin; for example, normal serum.

Third, the labeled antibody is eluted. The efficiency of elution may be increased by using heat treatment with a mild acid or a carrier; that is, an unlabeled antiserum. The carrier antibody prevents reabsorption of labeled antibody by blocking antigenic sites as rapidly as they are made free. The labeled antibody, thus prepared, is used to measure the combining capacity for red cells of sera or serum fractions, as illustrated in FIGURE 3.[14] A constant amount of red cells and labeled antibody is added to each of several tubes in relative amounts calculated to give approximately 70 per cent absorption of the labeled antibody. To the various tubes are added increasing amounts of a test serum. By blocking the antigenic sites of the red cell, the antibodies in this serum reduce the absorption of labeled antibody. An arbitrary combining unit was defined as that amount of test serum that will reduce by 50 per cent the absorption of labeled antibody by 0.5 ml. of an 0.4 per cent red cell suspension. Using this method of quantitating combining capacity, the distribution of hemolytic and combining activity was compared in the various globulin fractions that were separated by starch-block electrophoresis.[17] The results of several such experiments, one of which is illustrated in FIGURE 4, have demonstrated two peaks of antibody activity in the slow- and fast-moving γ-globulins respectively. While both these antibodies were capable of hemolyzing the red cells and both must

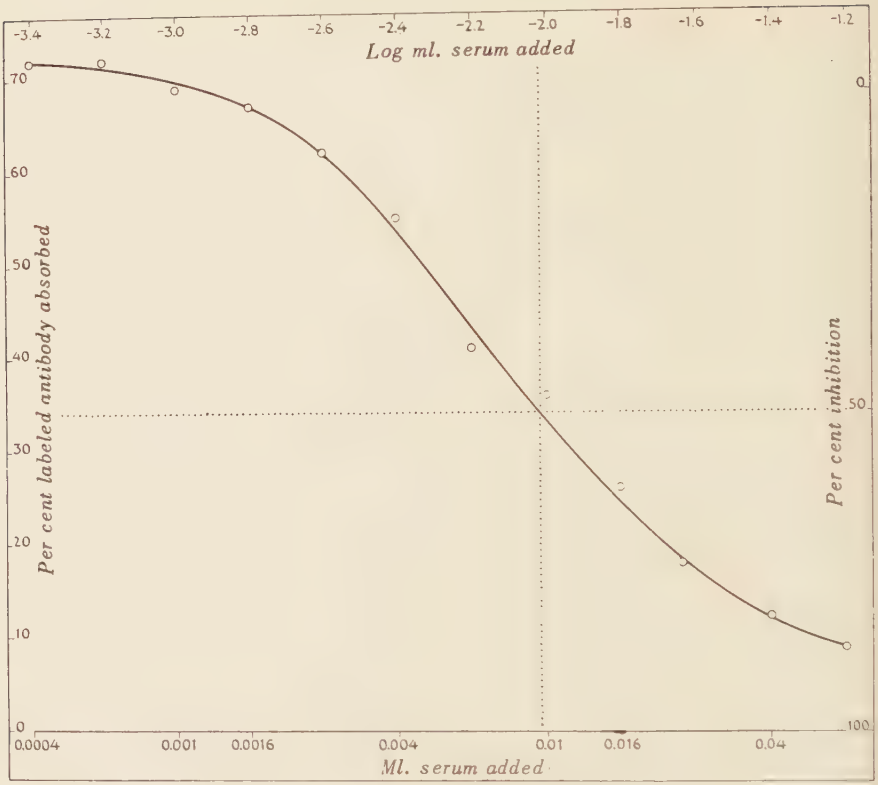

FIGURE 3. The effect of an antisheep-cell serum on the absorption by red cells of labeled antibody.

first combine with the red cell, the ratio of these 2 activities or the hemolytic efficiency of these 2 antibodies differed by a factor of 100. These experiments demonstrated that there is more to preparing the cell for hemolysis than union with antibody.

One of the factors responsible for the difference in hemolytic efficiency may be the relative sizes of the 2 antibody molecules. With antisera specific for both the protein and lipopolysaccharide antigens of the cell surface, the greatest hemolytic efficiency was always found in the most rapidly sedimenting fraction,[18] a fraction which has an estimated molecular weight of 900,000.

Existing evidence indicates that specificity for certain sites on the surface of the red cell is not a major factor in determining the hemolytic efficiency of an antibody. Antisera specific for either the protein or lipopolysaccharide antigens have been separated into fractions of high and low hemolytic efficiency. Experiments performed in the region of incomplete antibody absorption revealed no differences in the absorption of hemolytic and combining activity attributable to a difference in specificity. FIGURE 5 illustrates such an experiment performed with a serum of high hemolytic

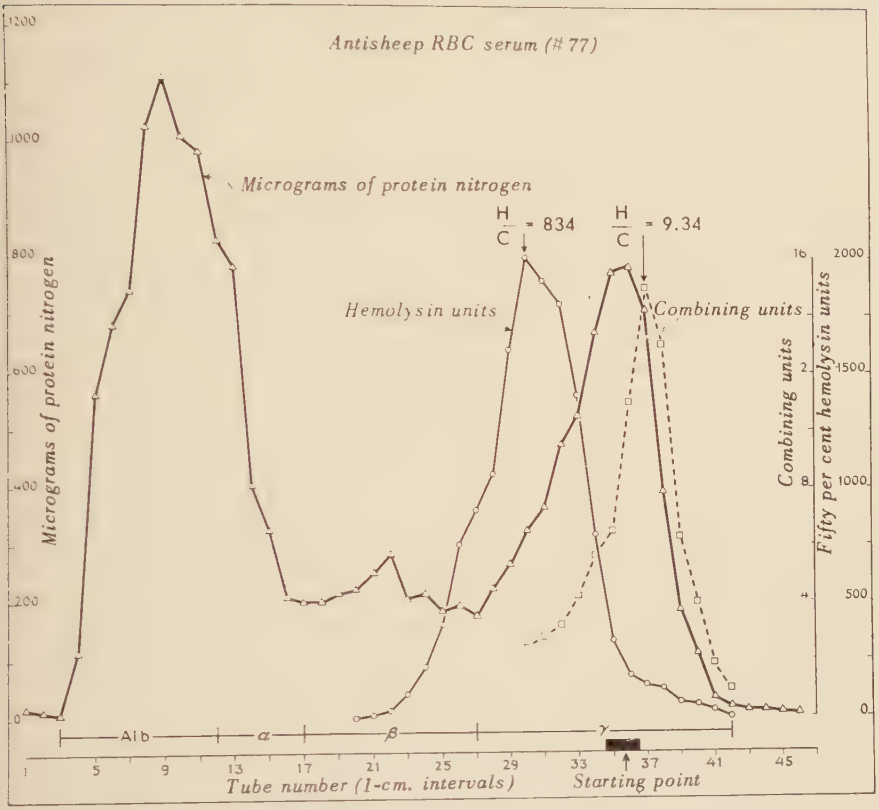

FIGURE 4. The separation by starch-block electrophoresis of 2 antired-cell antibodies that differ in their ratios of hemolytic activity to combining activity.

to combining ratio, a serum of low hemolytic to combining ratio, and a mixture of these 2 sera. A difference in specificity of the antibodies responsible for the 2 activities should give a proportionately smaller percentage of absorption of that antibody present in the relatively greater concentration. Actually, the reverse was found to be true. In the serum with the least relative amount of hemolytic activity, the absorption of this hemolytic activity lagged slightly behind the absorption of combining activity. This finding suggested that the hemolytically efficient antibodies were less avid for the red cell than were some of the other antibodies present in the serum. To determine the avidity of the hemolytically efficient antibodies, we separated labeled antibodies, the absorption curve for which indicated an avidity for red cells closely comparable to that of the hemolysin (FIGURE 6). These labeled antibodies could be separated from other more and less avid antibodies by a proper choice of the conditions of absorption and elution used in the separation process; for example, the amount of antigen, the temperature of washing and elution, and the time of elution. The half time of the union between the labeled antibody and the red cell was determined by a

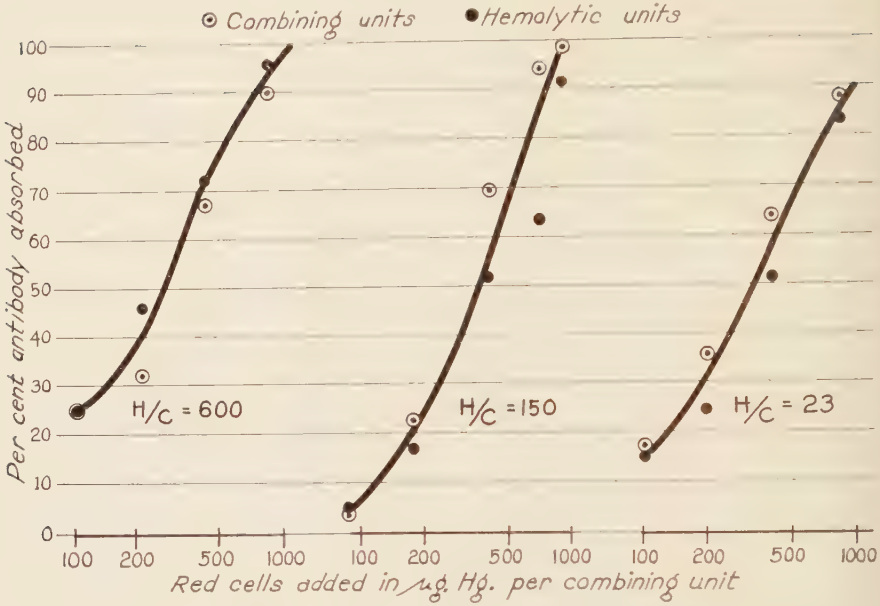

FIGURE 5. The absorption by varying numbers of red cells of the hemolytic activity and combining activity from a serum of high hemolytic efficiency (left), from one of low hemolytic efficiency (right), and from a mixture of the sera.

procedure similar to that used in the BSA-anti-BSA systems (compare FIGURES 2 and 7). Labeled antibody was absorbed to red cells, excess unlabeled antibody was added, and the time required for 50 per cent elution was determined. The half time of this antibody on the cell was found to be 3 min. Since trapping of the antibody in red-cell aggregates would slow down its elution, the figure of 3 min. should be considered an upper limit. This is still one sixtieth of the half time of the least avid of the anti-BSA antibodies studied. This demonstration of the relatively low avidity of hemolytic antibodies, confirming Bowman, Mayer, and Rapp's demonstration of the cell-to-cell transfer of hemolysin,[19] suggested the hypothesis that a relatively low avidity or a high turnover rate is a necessary property of hemolytically efficient antibodies. A possible scheme detailing this hypothesis may be represented as follows:

$$E + 2A \rightleftarrows A \cdot E \cdot A$$
$$A \cdot E \cdot A \leftrightarrows EA_2 \qquad (6)$$
$$EA_2 \xrightarrow{C'} \text{hemolysis}$$

In the first of these equations, the reaction between an erythrocyte (E) and 2 antibody molecules (A) results in the random distribution of the antibody molecules on the huge excess of antigenic sites available on the surface of the red cell. This reaction continues in equilibrium until, by chance, 2

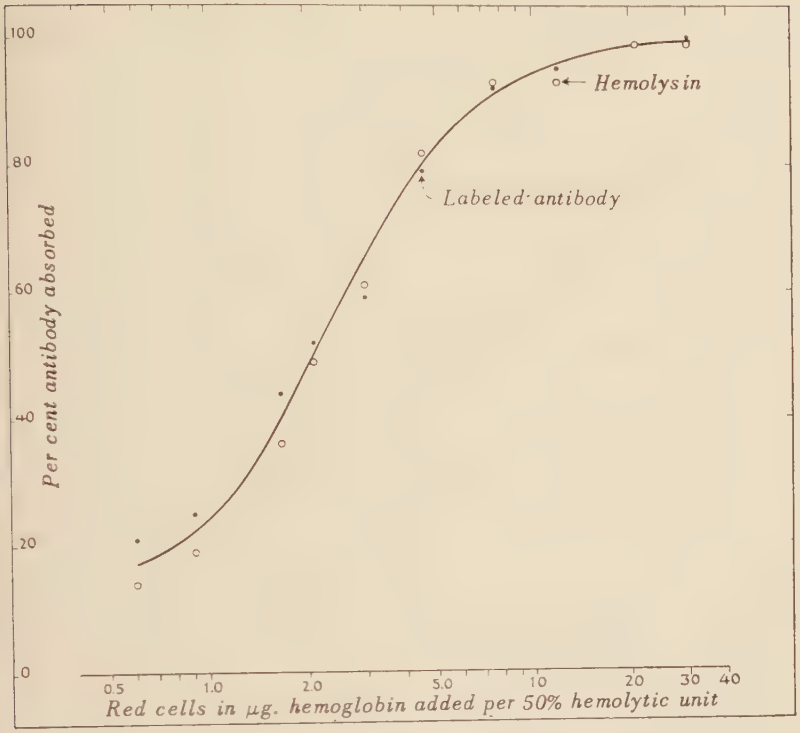

FIGURE 6. The absorption of hemolytic activity and radioactivity from an eluate by varying numbers of red cells.

antibody molecules combine with the red cell on adjoining sites to give the more stable complex EA_2. The rate of formation of EA_2 depends on the reversibility of the initial complexes. It is this complex EA_2 that is capable of fixing complement and hemolyzing the red cell.

This hypothesis of the mechanism of immune hemolysis is strengthened by the following considerations:

(1) An estimation of the number of antigenic sites per red cell and the number of hemolysin molecules per cell[14] requires that antibody molecules turn over once per minute to satisfy this hypothesis and experimental observations of hemolytic rate. The experimental finding of a half time of 3 min. (FIGURE 7) is considered a sufficiently close approximation.

(2) The hemolysis of the red cells, when complement is added before antibody, has been found to be a straight-line logarithmic decay[20] indicating a dynamic equilibrium in which the chance of hemolysis did not change with time. However, if antibody was added before complement, hemolysis proceeded rapidly at first and then slowed down until it reached a constant rate. The initial rapid rate would be expected from the above scheme because of the accumulation of cells with EA_2 complexes.

(3) Finally, the striking finding of Weinrach, Lai, and Talmage that

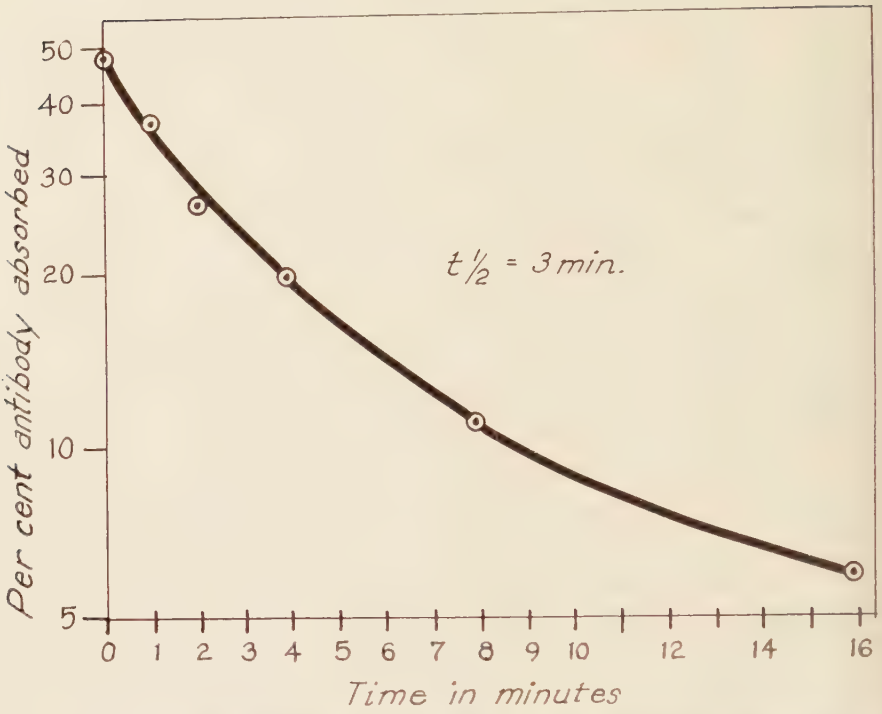

FIGURE 7. The half-binding time for red cells of labeled antired-cell antibodies, the absorption curve of which (FIGURE 6) was identical with that of their hemolytic activity.

the hemolytic rate is dependent on the square of antibody concentration,[20] strongly suggests a bimolecular antibody complex. Of equal interest, in this connection, was the finding of these authors that hemolysis with the smaller or less efficient molecule varied with the fourth power of the concentration of antibody. This finding suggests that different antibodies form different patterns on the surface of the cell, and that the type of pattern formed affects the hemolytic efficiency of the molecule.

In summary, the reaction between antigen and antibody is reversible, and the degree of this reversibility has important implications to the production of antibodies and to the mechanism by which antibodies manifest themselves as products of a biological activity. The value of the I^{131} label lies in the fact that it permits the quantitation of free and bound antigen and antibody and, thereby, a study of their primary reaction.

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STUDIES ON INSULIN LABELED WITH I^{131}

By Norman D. Lee

Radioisotope Service, Veterans Administration Medical Teaching Group Hospital and the Department of Chemistry, University of Tennessee Medical Units, Memphis, Tenn.

Introduction

Insulin is a protein hormone with a minimum molecular weight of approximately 6000, as determined by both chemical[1] and physical[2] methods. It is notable for its high content of cystine and for the complete absence of tryptophan and methionine.[1] The hormone isolated from beef, pork, or sheep shows small but distinct differences in amino acid composition; in addition, there is some evidence that the insulins from different species may also be immunologically distinct.[3] These amino acids are arranged in 2 chains, composed of 21 and 30 amino acid residues,[4, 5] that are probably of the α-helix type and connected by -S-S- bridges.[6] Different stereochemical representations have been proposed,[7, 8] but not proved, which agree that the *A* (21 residue) chain is bent and undergoes a change in the direction of twist in the region of residues 8 to 10. The molecule can complex with zinc and with certain other divalent cations to form a 12,000 molecular-weight (MW) unit that may then undergo aggregation to form dimers, trimers, and tetramers (pentamers have also been suggested),[9] depending on a variety of physicochemical influences. Concerning its state of aggregation in the plasma and tissues, however, there is no conclusive information and, mainly because of dilution, it is not entirely unreasonable to assume that insulin appears in the plasma as the 6000 MW unit. On the other hand, studies with fast ionizing particles have led to the suggestion that the dimer (24,000 MW) is the active form.[10]

Methodological Considerations

The effects of various physical and chemical agents on insulin have been studied intensively and have been reviewed recently.[11] It has been reported that iodine substitution occurs only on tyrosine residues, forming both monoiodotyrosine and diiodotyrosine, the histidine residues being unreactive.[12] Replotting some of the data of Fraenkel-Conrat and Fraenkel-Conrat[12] reveals that the extent of iodination determines the hormonal activity and that this relationship might be attributable to the formation of diiodotyrosine with little or no loss resulting from monoiodotyrosine formation (FIGURE 1). Extrapolation of these curves back to full activity suggests that, under conditions that could preclude the formation of diiodotyrosine, as much as 2 phenolic residues might be substituted with 1 iodine atom each per 6000 MW unit with no loss of biological activity.

Insulin has been labeled with I^{131} by a variety of methods, and all products have been reported to suffer no loss in potency.[13-18] The iodinations have been performed over a fairly narrow *p*H range, usually in a buffer on

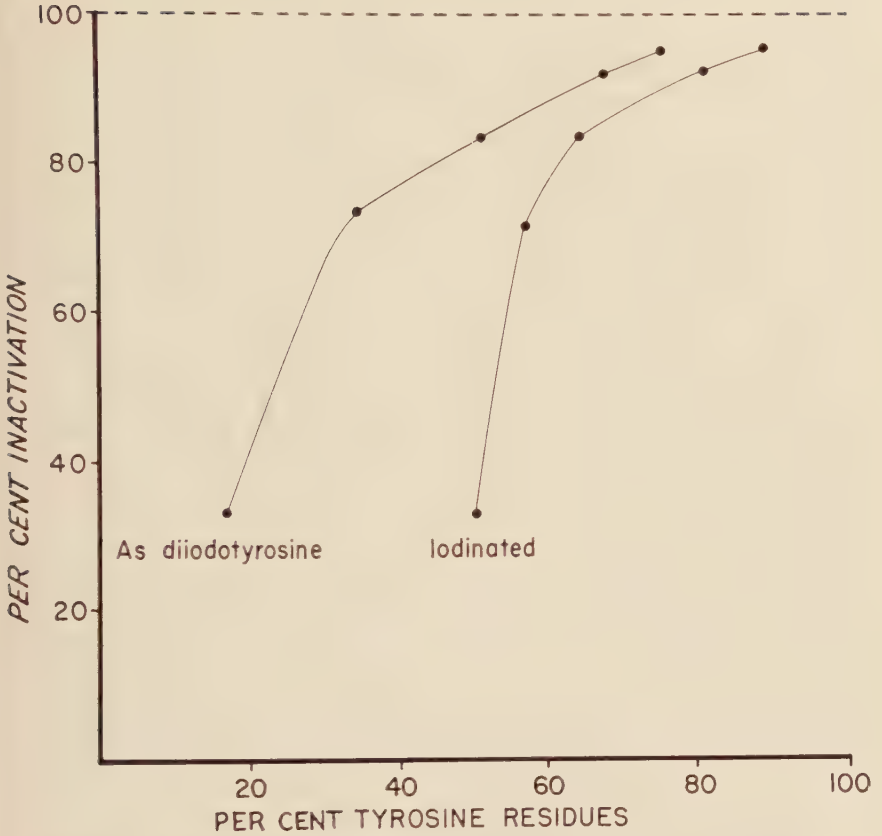

FIGURE 1. The relation between hypoglycemic potency and the extent of iodination of insulin. The curve for diiodotyrosine represents the percentage of the iodinated tyrosine residues that were disubstituted. (From the data of Fraenkel-Conrat and Fraenkel-Conrat.[12])

the alkaline side of the isoelectric point, the active agent being either I_2 or I_3^-. With some methods the active agent was generated from radioiodide by the use of an oxidant in the presence of the insulin; with others, it was separated in one form or another prior to coming in contact with the hormone. Purification from unbound I^{131} has been effected by dialysis, isoelectric precipitation, and filtration of the protein, and the resultant products have varied considerably in the degree of iodination, ranging in average values from a high of 1 iodine atom for each 6000 MW unit to a low of 1 in every 40. The term "average" is used here to emphasize the possible existence of multisubstituted and unsubstituted insulin molecules, although there is no direct evidence on this point. These materials were then tested for hypoglycemic potency and compared with the starting materials in order to establish the validity of their application as hormone tracers.

The criterion of hormonal potency is probably paramount in evaluating

these labeled preparations. Unfortunately, adequate testing is an involved and expensive procedure; hence, in the most precise sense, it cannot be said that exact bioanalytical proof of undisturbed potency has been generally obtained. Usually, however, comparable values for hypoglycemic potencies have been shown for starting materials and labeled products. More serious is the fact that, with sparse substitution—as low as 1 insulin molecule in 40 being iodinated –the bioassay really tested the hypoglycemic potency of the unsubstituted hormone. However, an extensive bioassay has been performed with insulin substituted with an average of 1 iodine atom per 6000 MW unit and revealed no loss in potency.[19] Consequently, on these grounds there is no reason to invalidate any preparation with a lesser degree of substitution.

The biological evaluation of insulin-I^{131} as a hormone tracer should include various aspects of its role in the body, since it is conceivable that other biologically significant functions may be altered in the face of undisturbed hypoglycemic potency. Insulin and insulin-I^{131} seem to be indistinguishable in connection with certain interactions with tissues. For instance, they compete equally for binding by the plasma of treated diabetics.[20] Furthermore, they compete equally for saturation of the inactivation systems *in vivo*[21] and as substrates for purified "insulinase" preparations *in vitro*.[16, 22] It has also been shown that a nonprotein "insulinase" inhibitor obtained from liver extracts inhibits the degradation of insulin-I^{131} both *in vivo* and *in vitro*.[22-24] In addition, it appears that both insulin and insulin-I^{131} are similarly bound to muscle *in vitro* and *in vivo*.[25] Finally, studies on the degradation of insulin-I^{131} in altered endocrinal states are consistent with the known physiological effects of insulin.[26, 27] Consequently, additional parameters indicate the biological identity of insulin-I^{131} with the starting preparations.

However, this resume is not without qualification since the starting materials themselves may not be identical to the endogenous hormone. Moloney and Coval[3] have prepared immune sera to insulin extracts obtained from various species, and have shown that in some cases the endogenous hormones did not seem to react with the sera as did the hypoglycemically effective antigens.

The central analytical problem is the identification of I^{131} radioactivity in tissues with the intact iodinated molecule. Direct deiodination of insulin-I^{131} does not seem to be a significant problem for studies lasting only a few hours, and the appearance of radioiodide in biological systems is coincident with inactivation and degradation of the insulin molecule.[16, 19, 20] Advantage has been taken of the precipitability of insulin-I^{131} by 5 per cent trichloracetic acid in the presence of carrier proteins. This permits a simple and rapid radioassay of tissue preparations, and it has been used, under proper conditions, to great advantage. It should be noted that certain real and possible breakdown products can be precipitated along with the I^{131}-labeled insulin.[28] The contamination of analytical samples by some of these products can be rendered insignificant by the use of proper technique; however, it should be clearly recognized that until the precipitable radioactivity can be extracted

from tissue and shown to possess physical and chemical properties similar to the labeled hormone, the use of this method provides only inferential data. This problem may be minimized by the zone electrophoresis methods developed by Berson and his co-workers.[17] Although these methods have been reported only in reference to insulin-I^{131} in plasma, they should be worthy of study in relation to other tissues. Similarly, paper chromatographic techniques developed for the study of insulin synthesis *in vitro*[29] might easily be applicable to the detection and assay of insulin-I^{131} in tissues.

The physical and chemical properties of labeled insulin have been studied only incidentally to other research. In view of the consequent paucity of this sort of information on I^{131}-labeled insulin and the bearing of this on both the nature of the labeling and the use and interpretation of the analytical techniques, a program has been initiated to study the physical and chemical properties of insulin-I^{131} labeled with an average of 1 iodine atom for each 6000 MW unit.

Investigative Applications

Berson and his co-workers[17] have reported that insulin-I^{131}, when administered intravenously to humans, is distributed rapidly into a volume apparently equal to 37 per cent of the body weight and is degraded at the rate of approximately 2 per cent per minute. It had been shown previously that sizable portions of injected insulin-I^{131} are distributed to a few specific tissues (FIGURE 2), and that some of these tissues actually may concentrate the labeled hormone to a considerable extent (FIGURE 3). This is true for both man and the lower animals.[19] Consequently, such apparent volumes and

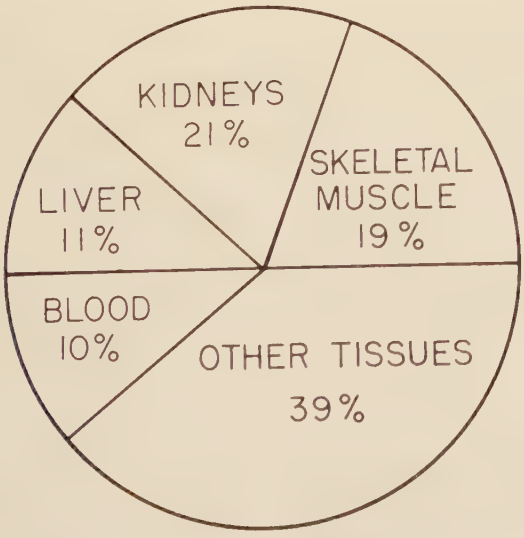

FIGURE 2. The distribution of total radioactivity in the rat 15 min. after the intravenous administration of insulin-I^{131}. Reproduced by permission from *The Journal of Clinical Investigation*.[19]

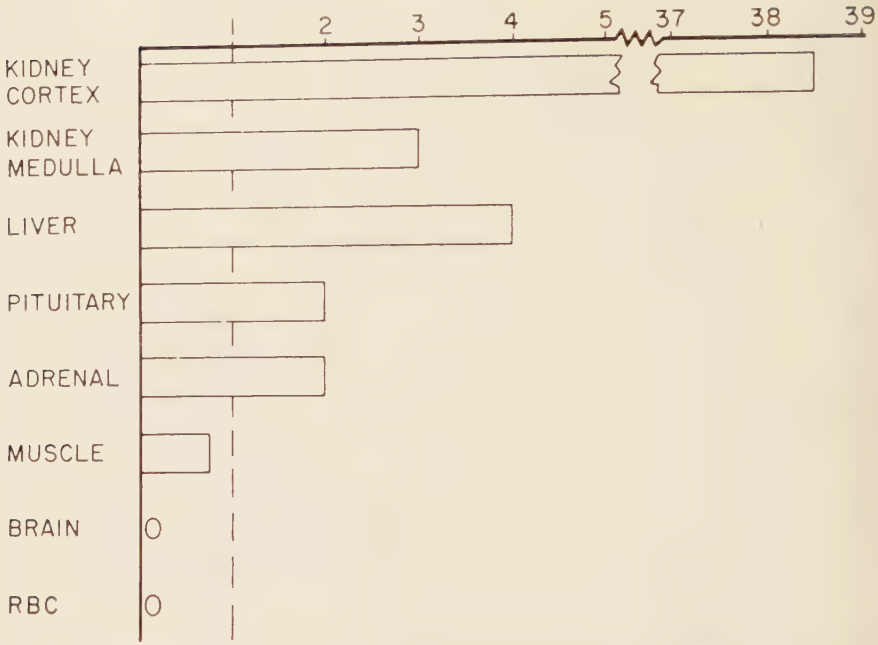

FIGURE 3. The concentration of radioactivity in various rat tissues 15 min. after the intravenous injection of insulin-I^{131}. Values greater than 1 on the horizontal scale (to the right of the vertical dash line) indicate the concentration of radioactivity to be proportionately greater than for uniform distribution throughout the body. Note that the value for kidney cortex necessitated disruption of this scale.

rates reflect weighted averages of the interactions of insulin-I^{131} with tissues. The removal of insulin-I^{131} from the blood, uncomplicated by these differential interactions, has been studied by Overman and his co-workers.[30] In this connection, these investigators had been studying the rate of disappearance of Na^{24} and K^{42} from the blood during the first few minutes after intrajugular injection.[31] Sampling from the carotid artery at 3-sec. intervals resulted in the curves shown in FIGURE 4; these may be more generally represented in FIGURE 5* The periodic phase arises from dilution of the injected mass into the circulating blood volume and takes no more than 30 to 40 sec. for completion. The early aperiodic phase is amenable to simple graphic and mathematical analysis (FIGURE 6) and may be represented by the equation $C/C_o = e^{-\lambda t}$, where C_o and C are blood concentration values at zero time and time t, respectively. Overman's group regards this phase as concerned with outward transcapillary movement, quantitatively uncomplicated by return from the tissues, and λ as a transport constant proportional to the rate of this movement and characterizing it. These studies have included a variety of substances for which λ values in the dog for Na^{24}, K^{42}

* The insulin-I^{131} used in the experiments reported in FIGURES 5 to 8 and TABLES 1 to 4 was prepared by Abbott Laboratories, North Chicago, Ill., from crystalline zinc insulin provided by Eli Lilly and Company, Indianapolis, Ind.

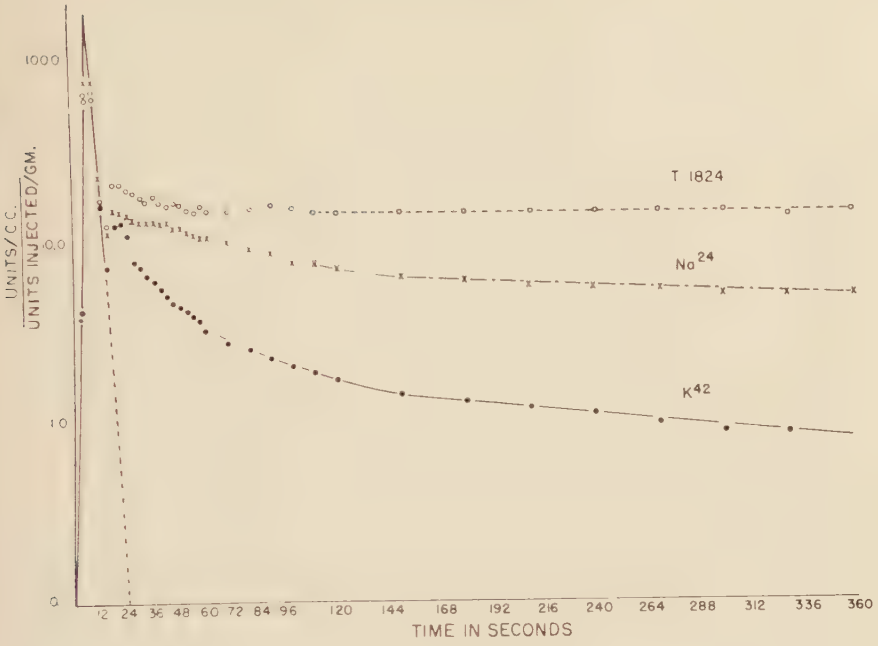

FIGURE 4. The disappearance of T-1824 (Evans' blue), Na^{24}, and K^{42} from the blood of the dog with respect to time.[30]

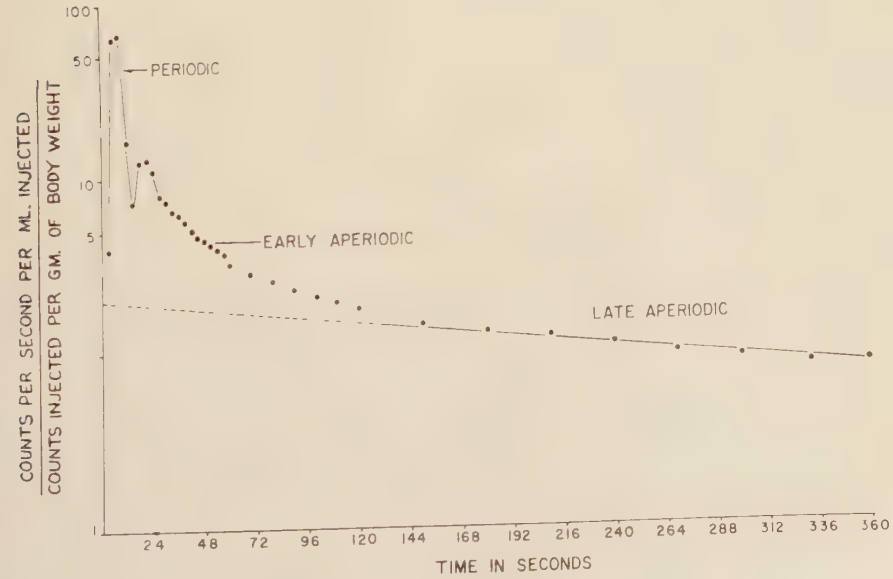

FIGURE 5. Generalized representation of FIGURE 4.[30]

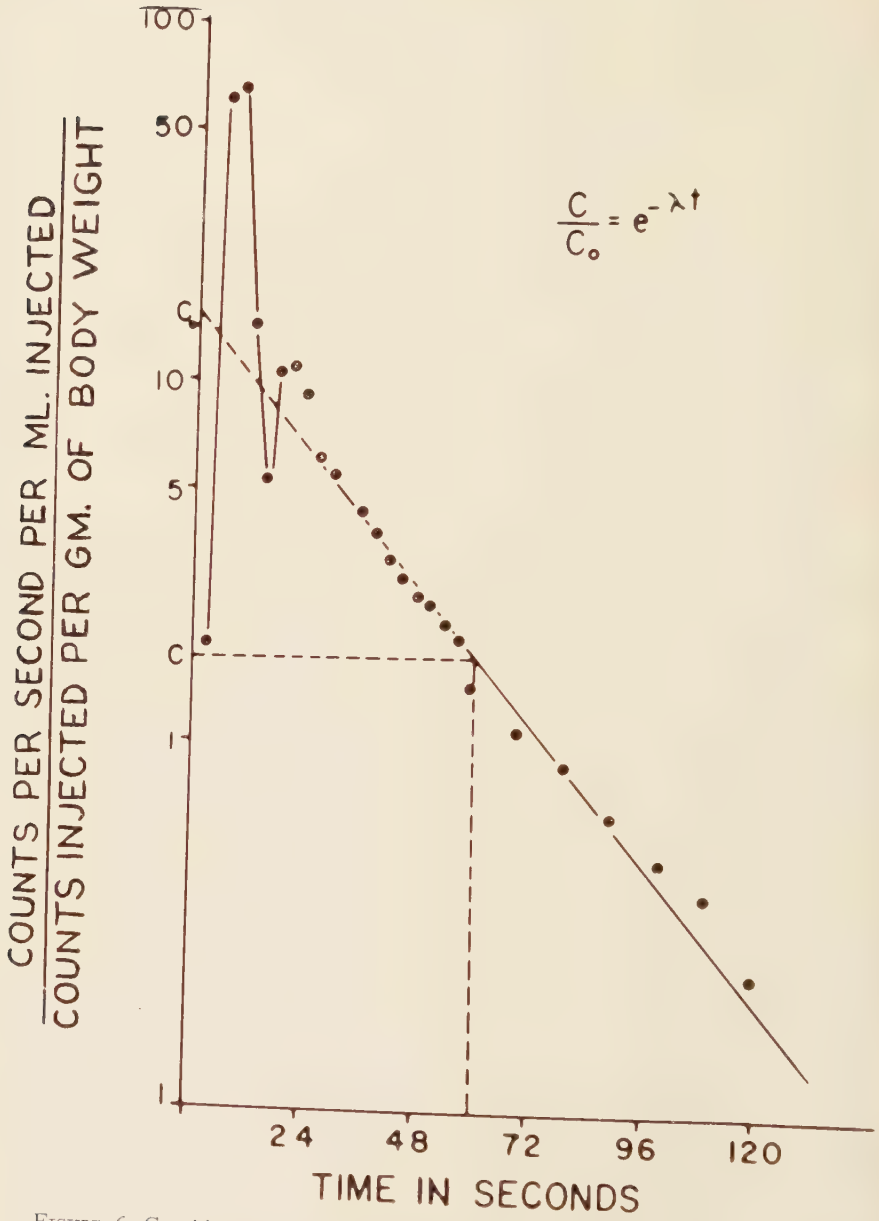

FIGURE 6. Graphical analysis for determination of transport constant for various solutes following intravenous injection. The points are determined as the differences between the measured values of the early aperiodic phase and the extrapolated values of the late aperiodic phase (FIGURE 3).

TABLE 1

THE "TRANSPORT CONSTANTS" FOR K^{42}, Na^{24}, AND INSULIN-I^{131}

	Transport constant	No. of animals
K^{42}	2.22 ± 0.39*	34
Na^{24}	1.38 ± 0.30	31
Insulin-I^{131}	1.34	5

*Mean ± standard deviation.

TABLE 2

THE ROLE OF MUSCLE AND KIDNEY IN THE REMOVAL OF INSULIN-I^{131} FROM THE PLASMA OF THE DOG

	Venous insulin-I^{131} conc., as per cent of arterial conc.			
Time after insulin-I^{131} injection, min.	3	5	15	30
Leg muscle	104	105	104	105
Kidney	80	78	90	

and insulin-I^{131} only are given (TABLE 1). It was rather surprising to find a value for the labeled hormone indistinguishable from that for Na^{24}, a small ionic molecule.[30] Some of the following information helps cast light on the interpretation of this finding.

The study and interpretation of both the early and late aperiodic portions of these curves suggest the possibility of a significant return of insulin-I^{131} from the tissues to the blood. This question is of particular importance in view of the known rapidity and firmness of insulin binding.[25, 32] TABLE 2 presents arteriovenous comparisons following intrajugular administration of insulin-I^{131} to the dog. It can be seen that throughout the interval under study the insulin-I^{131} concentration of the renal vein plasma was 10 to 20 per cent less than that of the arterial supply; this finding was expected in view of the extraordinary ability of the kidney to concentrate and bind insulin-I^{131} (FIGURE 3). The opposite finding for leg muscle can be interpreted only as resulting from the return of labeled molecules from some operationally defined compartment that became highly labeled during an earlier phase of distribution; this return became apparent due to the reversal of the specific activity gradient between the blood and the extravascular compartment in the muscle resulting from the removal of the labeled hormone from the blood, mainly by the liver and kidneys. Together with studies on the rapidity of the intracellular peneration of insulin-I^{131} into the liver and kidneys,[32, 33] these

data lend support to the belief that the transport constant value for this labeled hormone is related in some way to its transcapillary movement. Whether this represents an active and specific transport stands as an interesting speculation that is yet to be investigated.

The interactions of insulin-I^{131} with the various tissues occur subsequent to its removal from the blood; physiologically, these interactions are concerned with the metabolic functions and the inactivation of the labeled hormone. However, there must be physical contact prior to participation in these activities. It has been demonstrated by Stadie and his co-workers that both insulin and insulin-I^{131} are rapidly and firmly bound to various tissues both *in vivo* and *in vitro*.[25, 34, 35] Lee and Williams[32] showed that the binding of insulin-I^{131} in rat liver required structural integrity of the tissue and resulted in intracellular penetration and a characteristic distribution among the various subcellular structural elements. It is significant that there was very little binding of insulin-I^{131} to the submicroscopic microsomal fraction of kidney,[33] whereas considerable binding occurred to this fraction of liver. Furthermore, the amount of insulin bound per gram of liver was little reduced, if at all, by perfusion, and the distribution among the various subcellular structures was not disturbed. Hence, the magnitude and specificity of these interactions were considered to support the inference that these relationships were functionally concerned with the role of insulin. On the other hand, the action of insulin on the metabolic activities of liver has been reviewed critically by Levine and Fritz,[36] and the preponderance of evidence indicates that this hormone does not have an immediate and direct effect on this organ. Consequently, it is difficult to understand the quantitatively significant activity of the liver in concentrating and binding insulin, particularly since the hormone is secreted directly into the portal vein from the pancreas.

One function of the liver, however, is the degradation and inactivation of insulin and insulin-I^{131}. This organ and the kidneys are responsible for the major portion of the inactivation of this hormone; the other tissues are contributory only in a minor way.[36] Degradation is accomplished by means of a proteolytic enzyme system, referred to as "insulinase," that possesses distinct but not exclusive specificity for the hormone.[16, 22, 38, 39] The fact that this specificity is not absolute is hardly surprising in view of current knowledge of the structural basis for the specificity of proteolytic enzymes. It would be of considerable importance to know whether insulin bound to subcellular structures, particularly to the mitochondria and the microsomes, shows altered susceptibility to the action of this enzyme system which, in the main, is not associated with cellular structures.[40] The answer to this question would be of value in understanding the kinetics of the utilization of insulin in the whole animal and the significance of the liver activity in the concentration and binding of the hormone.

This question is still more important, since insulin does have a pronounced, although not immediate, effect on liver. This consists in a metabolic adaptation, which requires approximately 6 to 12 hours after the therapeutic administration of insulin to manifest itself, and in which various aspects of carbohydrate and lipid metabolism in the liver of the diabetic in-

sulin-treated animal return to prediabetic levels.[41] These changes coincide with the return of the elevated glucose-6-phosphatase activity of that organ to the lower normal values.[42] The net effect of a reduction in the activity of this enzyme system would be a diminution in this pathway of loss of carbohydrate potential so that proportionately more would be available for glycolysis and glycogenesis and those metabolic phenomena of a secondary nature. Consequently, the glucose-6-phosphatase system should be worthy of serious consideration as being causally related to those metabolic defects of the liver that are characteristic of diabetes.

Since glucose-6-phosphatase is almost exclusively associated with the microsomal fraction of liver,[43] the localization studies discussed above were reopened with a view toward establishing more clearly the specificity and significance of the intracellular distribution of insulin-I^{131}. Previous measurements had been compared and shown to be distinctly different from those for similar experiments[32] using iodide-I^{131}, thyroxine-I^{131}, and human-serum albumin-I^{131}. Nevertheless, it could not be convincingly argued that these comparisons indicated that the observations for insulin-I^{131} were specific for that molecule or were concerned with its metabolic function. Hence, we have carried out studies in which the structural or metabolic integrity of the insulin-I^{131} molecule has been altered in order to delineate its role in the liver more completely.

The only preparation to be presented in this report is insulin-I^{131}, completely inactivated by incubation with 0.033 M NaOH at 34° C. for 3

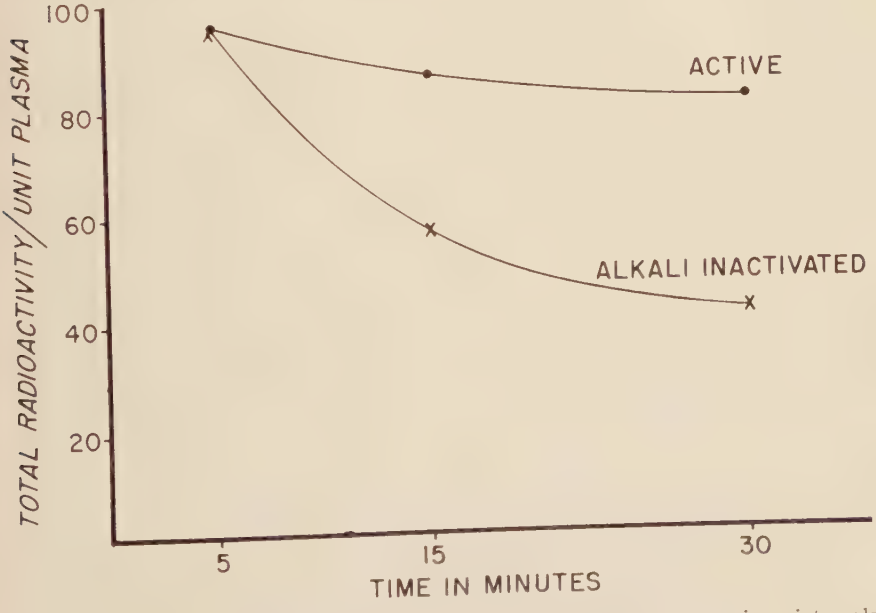

FIGURE 7. The concentration of radioactivity in rat plasma at various intervals after the intravenous injection of insulin-I^{131}.

hours.[44] This treatment results in the loss of small amounts of ammonia and sulfur.

Inspection of FIGURE 7 shows that alkali inactivation increased the rate of removal of insulin-I^{131} from the plasma, whereas similar data for the liver (FIGURE 8) showed a decreased rate of loss. These figures then indicate that the inactivation of alkali does not result in any loss in the ability of the liver to concentrate insulin-I^{131}, but that such insulin appears to be more resistant to those processes to which it is subject in the liver and hence is lost from that organ more slowly. From these observations, it seems reasonable to believe that the inactivation by alkali alters the susceptibility of insulin-I^{131} to degradation *in vivo;* experiments are being designed to examine this inference.

Underlying these changes are alterations in the nature of the binding of alkali-inactivated insulin-I^{131}. TABLE 3 presents the results of perfusion studies and shows that very little, if any, hormonally active insulin-I^{131} could be removed from liver, whereas alkali inactivation resulted in removal of the major portion of the hormone. In other words, the alkali inactivation of insulin-I^{131} does not appear to alter those properties of the hormone molecule concerned with its concentration by the liver, but does render less effective those properties concerned with the firmness of its binding to that tissue. The fact that the molecular aspects concerned with binding to tissue are altered is demonstrated in TABLE 4. Here it can be seen that the pattern of localization of insulin-I^{131} with respect to the var-

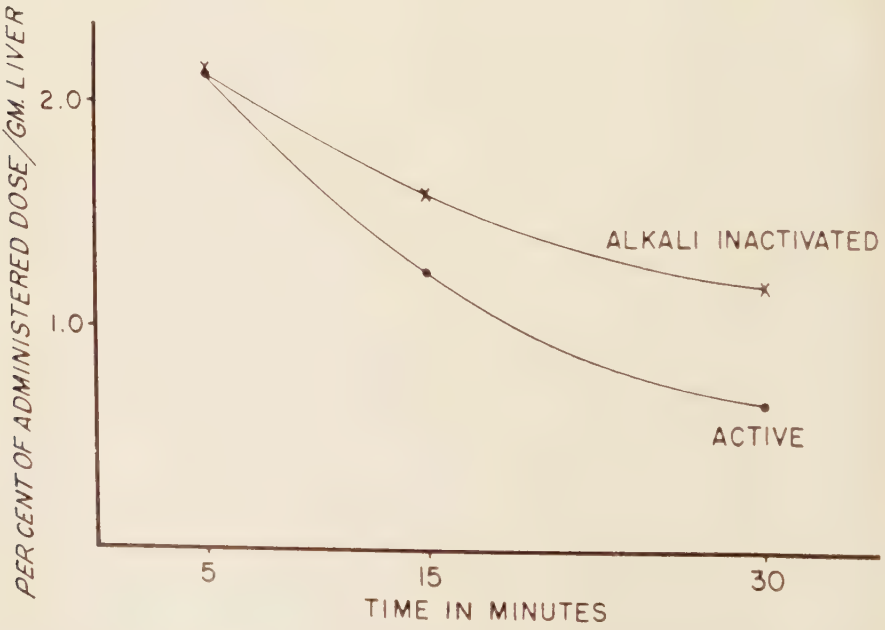

FIGURE 8. The concentration of radioactivity in rat liver at various intervals after the intravenous administration of insulin-I^{131}.

TABLE 3
THE CONCENTRATION AND BINDING IN INSULIN-I^{131} TO RAT LIVER

	Insulin-I^{131}, per cent of dose per gram of liver	
	Active	Alkali-inactivated
Before perfusion	2.1	2.4
After perfusion	1.8	0.9
Per cent lost by perfusion	14	63

TABLE 4
THE INTRACELLULAR DISTRIBUTION OF INSULIN-I^{131} IN RAT LIVER AT 5 MIN. AFTER ITS INTRAVENOUS ADMINISTRATION

Insulin	Active	Alkali-inactivated		
Preparation No.	4	4	5	5
Per cent dose/gram liver	1.75	0.50	1.06	*In vitro* addition
Intracellular distribution of radioactivity, %				
Nuclear fraction	22.5	16.5	14.4	15.1
Mitochondrial fraction	26.1	35.9	44.6	12.5
Microsomal fraction	24.4	10.7	8.8	3.0
Residual fraction	26.7	36.9	29.3	61.0
Number of experiments	6	6	6	2
Dose, μg.	2.9	2.9*	4.3	<0.1

ious intracellular organelles was altered markedly. Particularly notable is an increase in the proportion of the labeled hormone bound to the mitochondrial fraction and a proportionately greater decrease in the amount bound to the microsomal fraction. These effects have been seen with all inactivated materials prepared so far; the most strikingly consistent effect is the marked reduction in that portion of the insulin-I^{131} bound to the microsomal fraction.

These studies are being extended, particularly with respect to various other types of derivatives of insulin-I^{131} such as the active alkyl sulfate,[45] the active and inactive methyl esters[46] and, perhaps, xanthylinsulin.[47] However, the single altered insulin-I^{131} preparation studied thus far forms the basis for the following view. The hypoglycemic effect of insulin may be destroyed completely by relatively gentle treatment with mild alkali. This hormonal function does not seem to be concerned with those intramolecular

structures effective in the concentration of the labeled hormone by the liver; however, such molecular structures do appear to be operative in the firmness by which insulin-I^{131} is bound to the tissue of this organ, and may be concerned, in part at least, with the affinity of the hormone for specific intracellular organelles. The loss of hypoglycemic activity coincides with an altered binding of the hormone to the subcellular elements, the most notable of which are those concerned with the glucose-6-phosphatase activity, namely, the microsomes.

It has been suggested that, in the diabetic state, the correction of at least some defects in the liver metabolism of carbohydrates and lipids by insulin is determined by its binding to intracellular structures, particularly those which, upon homogenization, constitute the microsomal population. Indeed, it is a corollary of this hypothesis that such binding should represent a mechanism operative in the maintenance of the normal state. Certain findings with Orinase* in rats support this. This oral antidiabetic agent appears to be inoperative in the absence of functional pancreatic-islet tissue;[48, 49] however, the exact manner by which Orinase is effective as an antihyperglycemic agent remains unknown. Intravenous administration 30 minutes prior to the injection of insulin-I^{131} resulted in a shift in its intracellular localization in liver, exhibited as a disproportionate amount of the labeled hormone being bound to the microsomal structures. Consequently, this finding tends to support the contention that the action of insulin in the liver is related to its binding to cytostructural organelles and further justifies focusing attention on the microsomal fraction.

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DISTRIBUTION AND DEGRADATION OF HUMAN SERUM ALBUMIN LABELED WITH I^{131} BY DIFFERENT TECHNIQUES

By J. L. Steinfeld, R. R. Paton,* A. L. Flick,* R. A. Milch,† F. E. Beach

National Cancer Institute, Public Health Service, Bethesda, Md.

and D. L. Tabern

Abbott Laboratories, North Chicago, Ill.

Introduction

The use of I^{131}-labeled human serum albumin for biological studies has been subject to at least four criticisms.[1, 2] These may best be listed as related to (1) albumin, (2) stable iodine (I^{127}), (3) radioactive iodine (I^{131}), and (4) the preparation of iodinated albumin for human use. Let us consider them in that order.

(1) Albumin itself may not be a homogeneous entity, and albumin prepared for human use may differ from native human serum albumin as it exists within the body.

(2) The albumin will be denatured to a variable degree if iodination of the protein is excessive.[1]

(3) The millicuries of I^{131} added per mol of albumin and per milliliter of albumin solution may alter the degradation rate *in vivo* through self-radiation if over 50,000 roentgen equivalent physical (rep) are absorbed by albumin in a concentration of 5 mg./ml. or less.[3]

(4) The conditions of iodination of the albumin (*p*H, buffer, oxidizing agent, and temperature) and its preparation for human use (ion-exchange resin, dialysis, sterilization, conditions of storage, and concentration of albumin per ml. of solution) may alter some of the properties of the albumin.

Although the effects of these factors have been studied previously, the wide clinical use of radioiodinated human serum albumin (RISA)‡ prompted a systematic reinvestigation herein reported. Comparison of RISA with other I^{131}-albumin preparations was undertaken with respect to initial intravascular distribution, estimation of total exchangeable albumin, estimation of daily degradation rate, and presence of rapidly degraded I^{131} albumin in the material administered. Some of the variables in the preparations studied were self-radiation, the iodine-albumin molar ratio, and the use of iodine alone or of iodide-hypochlorite as the iodinating agents.

* Present address: Department of Medicine, University of Washington School of Medicine, Seattle, Wash.

† Present Address: Department of Surgery, Peter Bent Brigham Hospital, Harvard Medical School, Boston, Mass.

‡ Supplied by Abbott Laboratories, North Chicago, Ill.

Materials and Methods

Methods of iodination. All I^{131}-labeled albumins studied were prepared at Abbott Laboratories under the supervision of one of us (D. L. T.).

RISA. In the commercial preparation of RISA, serum albumin is diluted in bicarbonate buffer at *p*H 7.6 and placed in a reaction vessel equipped with a remotely controlled mechanical agitation device. A mixture of I^{131} and I^{127} is added on a molar basis such that 1 atom of iodine is present for each 5.5 molecules of albumin. This reaction is thoroughly cooled. Hypochlorite solution in an amount 4 times that calculated to liberate all of the iodide as iodine is added at intervals, with constant agitation. After standing overnight in the cold, the free iodide is removed on an ion-exchange column. The iodide-free eluate is diluted tenfold with normal saline, sterilized by filtration, assayed for radioactivity, and prepared for shipment.

The excess RISA preparation. A second test material in which the molecular ratio of iodide to albumin was 1:55 (instead of 1:5.5, as in RISA) was also prepared by the above method. This preparation will be called excess RISA.

The rapid-mixing RISA preparation. A third preparation was studied also; in this material, free iodine was used as the iodinating agent instead of an iodide-hypochlorite mixture. The technique of McFarlane[2] was followed except that bicarbonate buffer, rather than glycine buffer, was used along with rapid mixing in lieu of jet-iodination equipment; mixing was accomplished in a time similar to that used by McFarlane.

In vitro studies. At Abbott Laboratories, the most useful technique for determining relative amounts of tagged protein and uncombined iodide is ascending chromatography on paper with dilute methanol. Under these conditions the tagged proteins are precipitated at the point of application, and iodide has a partition coefficient (R_f) of approximately 0.85. As little as 1 per cent of iodide or iodate added to protein will be quite accurately detected by this technique. In an analysis of 22 consecutive lots of RISA tested by this procedure the average content of iodide was 0.6 per cent in the finished product after passage over an ion-exchange column. Only 1 lot of RISA showed as much as 1.1 per cent, while 10 showed no detectable iodide.

Dialysis of the 3 preparations against NaCl yielded less than 1 per cent of the radioactivity outside the dialysis sack when this experiment was continued for 6 hours at room temperature. Precipitation of the protein with 10 per cent trichloracetic acid in the cold also showed that 1 per cent or less of the radioactivity was in the supernatant layer.

In TABLE 1 can be found a comparison of the molar ratio of iodine to albumin in the preparations studied. In no instance was there more than 1 atom of iodine per molecule of albumin. There was considerable variation in the activity of the material expressed either as microcuries of I^{131} per milliliter of solution or per milligram of albumin. The microcuries of I^{131} per milliliter of solution were lowest in the rapid-mixing preparation, whereas the microcuries of I^{131} per milligram of albumin were lowest in the excess RISA preparation. RISA had the highest radioactivity, whether expressed as microcuries per milliliter of solution or as microcuries per milligram of

albumin. The rep delivered to each of the 3 preparations cannot be estimated from the final radioactivity per milliliter alone, because the final preparations were obtained by a variable dilution of the reaction mixtures. This will be discussed later.

TABLE 1

CHARACTERISTICS OF I^{131}-ALBUMIN PREPARATIONS

	RISA	Excess RISA	Rapid-mixing I
Mols iodine / Mols albumin	1:5.5	1:55	1:1
Radioactivity (μc./cc.)	700.0	235.0	75.0
Specific activity (μc./mg. albumin)	80.0	7.8	11.1
Albumin concentration (mg./cc.)	8.75	30.2	6.75

Assay of radioactivity. One *in vivo* and several *in vitro* counting systems were employed. Standards were counted in all systems so that intercomparisons could be made. *In vivo* radioactivity assays of the thyroid and thigh were made with a thallium-activated sodium iodide scintillation counter placed 20 cm. from the respective tissues. *In vitro* assays of serum, whole blood, and urine were carried out in a well-type scintillation counter. Duplicate samples of all *in vitro* specimens were counted to an accuracy of ± 3 per cent. A Drummond microhematocrit reading was obtained on the same specimen of oxalated blood where whole-blood radioactivity assay was employed.

Subjects studied. Nine patients at the Clinical Center of the National Cancer Institute were divided into 3 groups of 3 patients each. Every patient received each of the 3 preparations studied, so that each patient served as his own control (see TABLE 2). Since each group of patients received the

TABLE 2

SUMMARY OF COMPARATIVE STUDY OF I^{131} ALBUMINS

	Patient group A	Patient group B	Patient group C
Albumin preparation	RISA	Excess RISA	Rapid-mixing I
Albumin preparation	Rapid-mixing I	RISA	Excess RISA
Albumin preparation	Excess RISA	Rapid-mixing I	RISA

preparations in a different sequence, more rapid degradation of the preparations with time would suggest changes in biological behavior due to self-radiation during the 20-day storage period, although to be certain of this it would be necessary to give the same preparation repeatedly to the same individual.

The patients' diagnoses are listed in TABLE 3. Eight of the 9 patients were in a relatively stable state with regard to weight, dietary intake, serum albumin concentrations, and plasma volumes during the study period. N.E., a patient with chronic myelocytic leukemia, was in the terminal phase of her disease and received prednisone during the second and third periods of study. During this time her serum albumin concentration fell.

TABLE 3
LIST OF PATIENTS RECEIVING I^{131} ALBUMINS

Group	Patient	Diagnosis	Status	Treatment
A	N.E.	Chronic myelocytic leukemia	Blastic phase	None-Meticorten
A	S.H.	Chronic lymphocytic leukemia	Excellent	None
A	R.S.	Migraine	Excellent	None
B	M.B.	Malignant melanoma	Postoperative	None
B	J.A.	Esophageal carcinoma	Good	X ray
B	M.K.	Diabetes mellitus	Good	None
C	Stm.	Epidermoid carcinoma	Good	X ray
C	Carr	Epidermoid carcinoma	Good	None
C	Patt.	Psoriasis	Excellent	None

Procedure of study. For 2 days prior to and throughout the period of study each subject received 10 drops of Lugol's solution every 8 hours. Throughout the period of study, 24-hour urine specimens were collected in large bottles containing 10 ml. of a KI, $NaHSO_3$, NaOH carrier solution.* Stools were not collected since the daily I^{131} stool excretion is approximately 0.03 per cent, thus amounting to not more than 2 per cent of the administered dose during the entire month.[1, 4]

Patients were weighed daily before breakfast. Total caloric and protein intakes were calculated. Blood was drawn in the fasting state without stasis after the patient had been supine for at least 20 minutes.

In vivo radioactivity of the thyroid and thigh was assayed twice weekly to ascertain that there was no selective capture of I^{131} by the thyroid. Because of the difficulty of rinsing the calibrated syringes used for injection of I^{131} human serum albumin with venous blood, each study was begun by injecting 10 μc. of the measured I^{131} albumin into the rubber tubing of a slowly running normal saline infusion. The syringe was then rinsed 3 times, after which the infusion was stopped. Thus the patients received 40 ml. of normal saline along with the iodinated albumin. Blood samples were drawn without stasis from the opposite arm at 15 and 30 minutes for determinations of blood volume. Albumin and total protein concentrations were measured on the samples used for calculation of plasma volume. Values for the total exchangeable albumin and percentage of the dose excreted and

* Composition of carrier solution: 25 gm. potassium iodide, 44 gm. sodium bisulfite, 80 gm. sodium hydroxide in 100 ml. water. Stock solution is diluted 1:100 before use.

degraded each day were calculated according to the method outlined by Sterling[5] and by Berson *et al.*[1] In the second and third studies of each series, correction for excretion of residual I^{131} from the previous study was made from the daily degradation rate (for the last 4 days), from the percentage of the administered dose remaining in the body, and from physical decay of I^{131}.

Definitions

Terms used in the presentation of the results are defined as follows:

Rate = apparent instantaneous fractional rate.

Excretion rate = percentage of administered dose of I^{131} albumin appearing in the urine each day as I^{131}, corrected only for physical decay.

Degradation rate = the fraction $\frac{I^{131} \text{ excreted in urine} \times 100}{I^{131} \text{ albumin remaining in the body}}$

and therefore refers only to I^{131} albumin.

Results

TABLE 1 presents comparisons of the 3 preparations with respect to content of albumin, stable iodine, and radioactive iodine; however, since the original reactant concentrations in RISA manufacture are higher than those of the final preparations shipped, the self-radiation in rep for each albumin preparation at the beginning of each study period[6] is shown in TABLE 4. This table shows a twentyfold difference in radiation between the rapid-mixing iodine preparation, which received 9000 rep, and RISA, which received 200,000 rep at the time of the initial study. As stated previously, the rapid-mixing iodine preparation contained 7 mg. of albumin/cc. and the RISA 9 mg. of albumin/cc., so that they were comparable in this regard. The excess-RISA preparation with 30 mg. of albumin/cc. received an intermediate radiation dose of 40,000 rep.

TABLE 4

SELF-RADIATION IN REP TO I^{131} ALBUMINS

	RISA	Excess RISA	Rapid-mixing I
Study I	212,500	40,100	9,000
Study II	272,000	59,200	14,650
Study III	285,000	64,100	16,200

The results obtained in an individual patient are presented in FIGURE 1. The findings are characteristic of the other 8 patients, as well.

In FIGURE 1 it is shown that more of the RISA than of the other preparations was excreted* during the first day and that the cumulative excretion

* I^{131} was excreted by the patients and assayed in the urine and was used as the measure of degradation of RISA, excess RISA, or the rapid-mixing iodine preparation. To simplify presentation of the data we shall speak of excretion and/or degradation of RISA, excess RISA, and so on, although we did not, in fact, measure RISA excretion, but rather the excretion of I^{131} originally attached to the RISA.

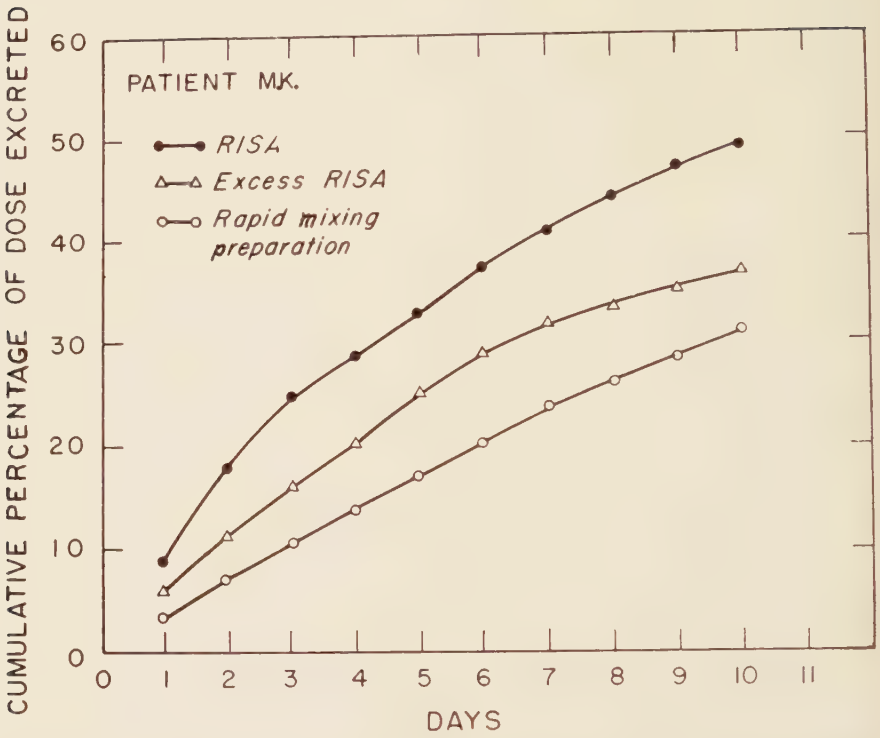

FIGURE 1. Comparison of the cumulative urinary excretion of I^{131}-labeled albumin preparations. The cumulative excretion of radioactivity in the urine, expressed as a percentage of the injected dose, is plotted on the ordinate, and is corrected for physical decay only. The time in days is plotted on the abscissa.

of RISA was always greater than that of the other 2 preparations, reaching 49 per cent on the 10th day. The excess-RISA preparation was excreted less rapidly, reaching 35 per cent of the administered dose on the 10th day. The patients, when they received the rapid-mixing iodine preparation, excreted 31 per cent during the first 10 days.

The data can be presented also by plotting the absolute percentage of the injected dose excreted each day. However, such a variable fraction of the preparations remains in the body that such a comparison loses much of its meaning. If the percentage of the remaining dose excreted each day (that is, the degradation rate) is plotted, it is possible to make a more meaningful comparison of these preparations. This is shown in FIGURE 2, in which the ordinate represents the degradation rate and the abscissa indicates the time in days. The degradation rate is the ratio of the percentage excreted each day to that remaining in the body on that day. In this instance a distinct difference also emerges. While the degradation rate appears to be approximately the same for all 3 preparations between days 6 to 10, in the first few days considerably more RISA than rapid-mixing preparation is degraded. This pattern was similar in all 9 patients.

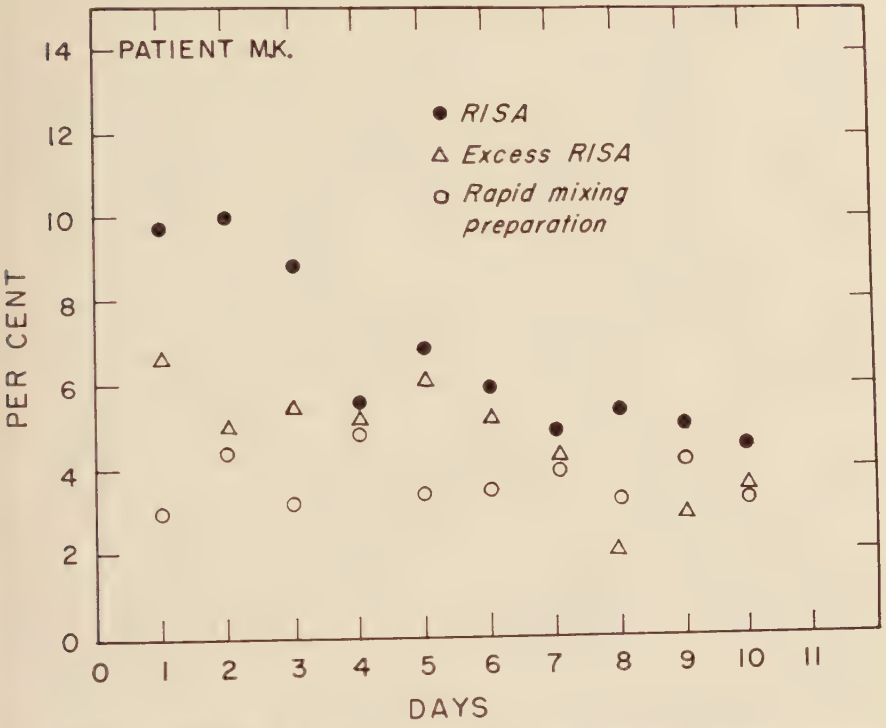

FIGURE 2. Comparison of the daily degradation rates of three I^{131}-labeled preparations. The degradation rate is plotted on the ordinate. The time in days is plotted on the abscissa.

TABLE 5 shows the 10-day cumulative I^{131} excretion when all 9 studies using a single preparation were averaged. The average 10-day cumulative excretion of I^{131} was 45 per cent in these 9 patients when they received RISA, 37 per cent when RISA was prepared with an excess of albumin, and 31 per cent when free iodine was used as the iodinating agent and rapidly mixed with the albumin. That these averages are representative of the 3 studies is shown in TABLE 6, which gives the 10-day cumulative excretion of I^{131} by groups. In each group the cumulative 10-day excretion of I^{131} was least with the rapid-mixing iodine preparation, intermediate with the excess albumin preparation, and greatest with RISA. Furthermore, the temporal sequence of increasing exposure to radiation also can be investigated in TABLE 6. Patients in Group A who received RISA excreted 44 per

TABLE 5

AVERAGE 10-DAY CUMULATIVE I^{131} EXCRETION FROM I^{131} ALBUMIN

RISA	45%
Excess RISA	37%
Rapid-mixing I	31%

cent of the dose injected, while those who received RISA in another 10 days excreted 48 per cent, but the patients who still received the RISA 10 days later excreted 42 per cent of the injected dose. Because these are 3 different groups of patients, this finding might represent different average rates of degradation; in fact, all of the I^{131} albumins were deteriorating slowly with time. Careful scrutiny of TABLE 6 cannot support the idea that, in this experiment, the rep accumulated by the albumin while on the refrigerator shelf affected its biological behavior. Damage was largely completed at the time of the initial study. In all 3 instances less of the rapid-mixing preparation than of the others was excreted.

Moving now from the percentage of the original dose excreted to the percentage of the remaining dose excreted, that is to say, the degradation rate, TABLE 7 gives the individual daily results for Study 3. In this mass of num-

TABLE 6

TEN-DAY CUMULATIVE I^{131} EXCRETION BY GROUPS

Patient group A		Patient group B		Patient group C	
RISA	44.3	Excess RISA	41.1	Rapid-mixing I	31.8
Rapid-mixing I	32.9	RISA	48.1	Excess RISA	35.5
Excess RISA	39.3	Rapid-mixing I	30.5	RISA	41.5

TABLE 7

STUDY 3: PERCENTAGE OF I^{131} ALBUMIN DEGRADED PER DAY

	Excess RISA			Rapid-mixing I			RISA		
Day	N.E.	S.H.	R.S.	M.B.	J.A.	M.K.	Stm.	Carr	Patt.
1	12.5	5.3	6.9	4.0	3.8	3.0	9.9	13.4	6.7
2	13.0	6.0	11.8	5.6	4.8	4.4	4.6	5.9	6.5
3	17.9	5.8	6.7	4.5	3.6	3.2	2.6	5.0	6.7
4	9.8	3.7	4.6	4.2	5.2	4.9	3.3	4.6	6.1
5	11.9	3.7	5.0	3.4	3.6	3.6	3.0	3.5	5.0
6	10.1	3.8	4.8	3.6	3.5	3.5	3.0	4.4	5.0
7	13.6	5.0	3.3	3.2	3.6	4.1	3.7	3.8	5.4
8	8.8	3.6	3.2	2.9	4.7	3.4	2.6	3.5	4.2
9	8.1	4.2	4.1	2.8	3.1	4.2	3.0	3.0	5.8
10	8.0	4.1	2.4	3.1	1.9	3.4		4.2	4.9

bers attention is focused immediately on patient N.E., who had a most rapid rate constant through the period of study. This patient, in the terminal phase of chronic myelocytic leukemia, became febrile, but without evidence of systemic infection, and was treated with 200 mg. of prednisone (Meticorten) daily during this last phase of the study. In the other patients the pattern seen in the graph presented earlier occurs; namely, an early increased degradation rate in patients receiving RISA or the excess-RISA preparation. This is most marked in those receiving the former preparation.

Since the higher degradation rates were most pronounced on days 1 and 2, these days may be omitted for purposes of comparison. The average rates for days 3 through 10, omitting those of N.E. on the second and third studies, are shown in TABLE 8. The omission of days 1 and 2 still does not give uniform rates of degradation for the 3 preparations: the rapid-mixing preparation giving the lowest daily rate (3.6 per cent), the excess RISA being degraded at 4.4 per cent per day, and RISA itself at 4.7 per cent per day.

TABLE 8

AVERAGE PERCENTAGE OF I^{131} ALBUMIN DEGRADED PER DAY FROM 3RD TO 10TH DAYS

RISA	4.7
Excess RISA	4.4
Rapid-mixing I	3.6

TABLE 9

AVERAGE PERCENTAGE OF I^{131} ALBUMIN DEGRADED DURING DAYS 1 AND 2, COMPARED WITH DAYS 3 THROUGH 10

RISA	4.4% more
Excess RISA	2.3% more
Rapid-mixing I	0.4% more

Another comparison that immediately suggests itself is that between the rate on days 1 and 2 and that on days 3 through 10. In TABLE 9 it is seen that, of the RISA, 4.4 per cent per day more was excreted on days 1 and 2 than on days 3 through 10, when all 9 studies were averaged. However, 2.3 per cent more of the excess RISA preparation and only 0.4 per cent more of the rapid-mixing iodine preparation were likewise degraded more rapidly.

These patients were in a reasonably stable state, and the following values (TABLE 10) were obtained for blood volumes in patient Group A. Groups B and C showed greater consistency with respect to red-cell mass and plasma volume. This group was chosen for presentation because of its variation in plasma volume, which was not characteristic of other patients studied. The larger plasma volume occurred when patient R.S. received the excess-RISA preparation, degradation and excretion of which were intermediate between the other 2 preparations. Everything considered, there was a remarkable correlation in red-cell mass and plasma volume from 1 preparation and 10-day period to the next.

A comparison of total exchangeable albumin as estimated by the isotope dilution technique in Group A is shown in TABLE 11. The values for total exchangeable albumin agree well within 7 per cent, the variation being related more to changes in serum albumin concentration between studies

TABLE 10
BLOOD VOLUMES—PATIENT GROUP A

	S.H.	N.E.	R.S.
	RISA		
Total blood volume (cc.)	5463	3439	4334
Red-cell mass (cc.)	1945	751	1696
Plasma volume (cc.)	3514	2688	2638
	Rapid-mixing I		
Total blood volume (cc.)	5415	3269	4596
Red-cell mass (cc.)	1945	775	1626
Plasma volume (cc.)	3470	2494	2968
	Excess RISA		
Total blood volume (cc.)	5467	3237	5077
Red-cell mass (cc.)	1880	738	1687
Plasma volume (cc.)	3587	2499	3390

TABLE 11
TOTAL EXCHANGEABLE ALBUMIN (GM.), GROUP A

	N.E.	S.H.	R.S.
RISA	195	327	311
Rapid-mixing I	171	303	281
Excess RISA	115	321	316

than to the final iodinated human serum albumin (IHSA) distribution. The average variation in total exchangeable albumin was about 7 per cent. Group A was chosen for presentation again because the variation was greatest in this group and because the findings in patient N.E. were interesting in and of themselves. This patient had a remarkable fall in total exchangeable albumin associated with a high daily rate of degradation.

Discussion

The marked difference between the RISA and the rapid-mixing iodine preparation could be caused by 3 known factors or perhaps by others still unknown. A hypochlorite-iodide mixture was the iodinating agent in the manufacture of RISA, as compared with iodine in the rapid-mixing iodine preparation. RISA's self-radiation was 200,000 rep compared to 9000 for the rapid-mixing iodine preparation. Finally, in the manufacture of RISA, iodination is slow through the gradual addition of aliquots of hypochlorite; in the rapid-mixing iodine preparation the opposite is true. The present study gives no information regarding which of the above 3 factors is important or most important in the preparation of I^{131} albumin, but the intent of

the experiment herein reported was to compare compounds that, by general agreement, would be considered other good HSA preparations, with RISA.

The use of iodine alone as the iodination agent does not result in a lack of oxidation of the sulfhydryl groups of the protein. As was shown some years ago, both iodination and oxidation proceed concurrently, at least under all conditions tried.[7] The use of an oxidizing agent such as hypochlorite, therefore, cannot be indicted as altering the protein on that basis alone. The fact that the same method of iodination, with an oxidizing agent but with more protein, resulted in a preparation with less of the rapidly degraded components suggests that the excess of oxidizing agent may have altered some of the protein molecules. However, we have shown in TABLE 4 that there was also a fivefold difference in self-radiation between the excess-RISA and RISA preparations.

It is pertinent to note at this point that Yalow and Berson[3] have shown that 50,000 rep administered to a 0.2 mg./cc. albumin solution did alter the rapidity with which the I^{131} tag was lost from the albumin.

With reference to the mixing problem, the feeling at Abbott Laboratories is that slow addition of reagents with cooling has as sound a chemical basis as any other known procedure.

Considerable evidence indicates that the presence of about 10 iodine atoms per molecule of albumin produces no change in the protein as studied by electrophoresis or in the ultracentrifuge:[1, 5] when, accordingly, one further decreases the ratio of iodine to albumin to 1:5.5, it is unlikely that more than 1 per cent of the albumin molecules will have more than 5 iodine atoms on a statistical basis, and it is therefore unlikely that the molar ratio of iodine to albumin will, in itself, affect the biological behavior of the protein.

Of the factors that might influence behavior of I^{131}-labeled human albumin *in vivo*, at least 1 is at present beyond our control. The rules of the Division of Biologic Standards of the National Institutes of Health, which supervises the distribution of blood and blood products, preclude the use, in human beings, of material not made from protein isolated by standard methods known to minimize the possibility of the presence of hepatitis virus. In practice, the material must be made from a pooled lot of blood and be isolated by the Cohn method, using ethanol precipitation at low temperatures. This automatically rules out certain nonethanol-treated fractions as used by McFarlane.[2]

It should be noted that, in the triple study reported, all 3 of the preparations gave values for red-cell mass and plasma volume that were within the limits of changes that might occur in 10 days. Since RISA has been used most frequently for determinations of blood volume, this finding is a reassuring one.

In the actual use of I^{131} albumin in a biological study for the estimation of the biological half life of albumin, it is necessary to collect specimens for from 30 to 40 days. It must be emphasized that the comparative study herein reported yields data on immediate intravascular distribution and total exchangeable albumin (assuming equilibration in body albumin pools to have occurred by the 10th day). No absolute figure for the rate of albumin

metabolism can be given when patients are studied for only 10 days, but comparisons of I^{131}-albumin preparations can certainly be made in such a period of time. In fact, other reported studies and our own work indicate that, if the rate of degradation of I^{131} albumin in a reasonably normal individual does not exceed 6 per cent on days 1 or 2, the preparation may be assumed to be a relatively good one.

Summary

(1) Three I^{131}-albumin preparations have been compared with respect to self-radiation from I^{131}, concentration of stable albumin, and oxidizing agent used for iodination.

(2) Each of the 3 preparations studied yielded comparable values for blood volume and total exchangeable albumin.

(3) I^{131}-albumin preparations with rapidly degraded components on days 1 and 2 continue to give high values for albumin degradation rate as late as the 10th day.

(4) Self-radiation of I^{131} albumin at a level of 200,000 rep in a concentration of 10 mg./cc. alters the rate at which the I^{131} tag is lost from the protein.

(5) This effect can be decreased by lowering the self-radiation to 40,000 rep, and it can be decreased even further by lowering the exposure to 10,000 rep.

(6) The use of a mild oxidizing agent such as hypochlorite in HISA preparation may alter the protein slightly as compared to the use of iodine as the oxidizing agent.

Addendum

Once the results were tabulated, the need for further studies became obvious. Thus, D. L. Tabern of Abbott Laboratories made some 10 other planned preparations, of which 8 were sent to the Clinical Center of the National Cancer Institute. At least 5 patients received each preparation. The self-radiation in rep was less than 10,000 in all preparations studied.

A first important finding was that it was not possible to prepare the rapid-mixing iodine preparation with any reasonable approximation of yield. This is apparently due to the variable concentration of reducing agent (bisulfite) added to iodide^{131} at the Atomic Energy Commission's installation at Oak Ridge, Tenn. A few years ago the concentration of bisulfite could be approximated on the assumption that the solids in parts per million on the I^{131} shipping slip all represented bisulfite. However, this apparently is no longer the case, and the reducing agent poses a real problem. One answer was to distill the radioactive iodine into the albumin solution. Four such preparations were studied; 2 of these were prepared from a lot of commercial albumin different from that used by Abbott Laboratories for all the other preparations and for RISA manufacture for the last few years. The latter 2 preparations, using a different lot of commercial albumin, gave values for blood volumes which were 4 to 5 times too high, and these preparations were quantitatively excreted within 72 hours. It is not known

whether the alteration of the protein occurred before or after iodination, but this question is under study. The other 2 preparations made using iodine as the oxidizing and iodination agent gave an excretion and degradation pattern similar, not to that of the rapid-mixing iodine preparation, but to that of RISA, in spite of the fact that these preparations had a self-radiation of less than 10,000 rep.

In addition to the above 4 preparations, 4 RISA-type preparations were also studied. These were made under the rigid application of all conditions of RISA manufacture, except that less than 10,000 rep of self-radiation were received by the albumin at the time the patient studies began. These RISA-type preparations gave an excretion and degradation pattern almost intermediate between those of excess RISA and rapid-mixing iodine reported in this study.

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DISTRIBUTION DYNAMICS OF CIRCULATING AND EXTRAVASCULAR I^{131} PLASMA PROTEINS*

By David Gitlin

Department of Pediatrics, Harvard University Medical School, and the Children's Hospital, Boston, Mass.

Introduction

The use of radioiodine, specifically of I^{131}, as a label for plasma proteins has provided a tool for the study of plasma protein metabolism that for the following 5 reasons is unique: (1) the label is relatively inexpensive; (2) it is easily incorporated into a plasma protein that has been isolated and purified independently; (3) it can be given safely to human beings; (4) the labeled protein behaves and is handled by the body in a manner indistinguishable from that of the native protein, at least up to the process of degradation; and (5) here the departure of behavior from that of the native protein is likewise advantageous, since the labeled products of degradation are not reincorporated, but are excreted. Moreover, I^{131} labeling can be used in conjunction with another tool that has proved very useful in the study of the metabolism of plasma proteins; namely, immunochemistry. With suitable antisera, specific plasma proteins may be estimated easily and accurately, supplementing the quantitative data obtained with I^{131} tracers.

This paper is a discussion of some aspects of the metabolism of plasma proteins that have been studied through a combination of immunochemistry and I^{131} labeling of specific plasma proteins.

Method of Iodination

In the preceding papers it has been emphasized repeatedly and justifiably that iodination procedures, as well as radioactivity itself, may induce serious physicochemical or biochemical alteration of plasma proteins. It is pertinent to the general theme of caution that has been stressed in this monograph to note the methods of preparation of the iodoproteins used in the studies reported here. In brief, the following points are of importance: (1) The use of all organic solvents was avoided assiduously, except for that of ethanol under carefully controlled conditions for the isolation and purification of specific plasma proteins prior to iodination. (2) The *p*H during iodination was controlled carefully. (3) No more than 3 atoms of iodine were bound per molecule of plasma protein, and usually less than 2. (4) Suitable amounts of carrier protein were always added to the iodo-

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proteins as soon as the latter were prepared. (5) The labeled proteins were administered to their recipients within 24 hours of the time of their preparation.

Our experiences with immunochemistry, fractionation, and iodination have taught us that good general technique, applied with a gentle hand, is necessary at all times. Otherwise, denaturation, not only of the labeled product but, of equal importance, of the isolated purified plasma protein, may result even before iodination. In the case of certain labile proteins, such as the lipoproteins, the entire process, from the time when blood was obtained from donors for the isolation of the pure protein to the moment when the labeled material was injected into the recipients, was performed within 24 to 72 hours.

The actual iodination procedure was simple. I^{131} was obtained carrier-free as sodium iodide in sodium bisulfite. The iodide and bisulfite were oxidized with nitrous acid, and the excess acid was neutralized with sodium hydroxide.[1] Carrier KI_3 was added, either prior to the oxidation (in which case the KI_3 contributed carrier I_2), or after the oxidation step.[2] The resulting I_2 or KI_3 solution was then mixed with the plasma protein to be labeled, the latter being dissolved in a bicarbonate buffer of *p*H 9.5. The labeled proteins were dialyzed for from 24 to 72 hours against changes of isotonic potassium iodide and sodium chloride. Carrier protein, usually albumin, was added before or after dialysis to a concentration of about 1 gm. per cent. Free or loosely bound radioactivity was determined by precipitation of the labeled protein with trichloroacetic acid, or with specific antibodies, or by dialysis. In no instance was a protein used if the free or loosely bound radioactivity was more than 2 per cent of the total radioactivity of the preparation. The efficiency of iodination by this method was very low as compared with those reported by others, being only about 15 per cent; part of this low efficiency, of course, was due to the utilization of I_2 or I_3^- for the oxidation of -SH groups on the plasma protein. This relative inefficiency was more than outweighed by a minimum of alteration of the plasma protein and the relative inexpensiveness of I^{131} when used on a medical research scale.

Dynamics of Distribution of Plasma Proteins in the Human Body

Dynamic equilibrium. As we are all well aware, when such radioiodinated plasma proteins are given intravenously to normal human beings, the plasma concentration of the tracer follows a very definite pattern with time.[3-5] Initially, the concentration of labeled protein in the plasma falls relatively rapidly and then more slowly, finally assuming a phase wherein the fractional rate of disappearance is a constant, or the decline in concentration is logarithmic with time. The logarithmic phase of disappearance, of course, reflects the fractional rate of catabolism, or that fraction of the body pool of the protein catabolized per unit of time, as judged from the appearance of labeled degradation products in the urine, the blood, and the tissues. On the other hand, the initial rapidly falling phase of the plasma disappearance curve is the result of at least two factors: (1) catabolism which,

of course, begins almost at zero time, and (2) diffusion of the labeled protein from the vascular system (FIGURE 1).

Associated with the initial phase of the plasma disappearance curve is the appearance of the plasma protein in the interstitial fluid of the tissues, as one would expect. For example, in children with congenital agammaglobulinemia, γ-globulin is virtually absent, not only from the blood, but from

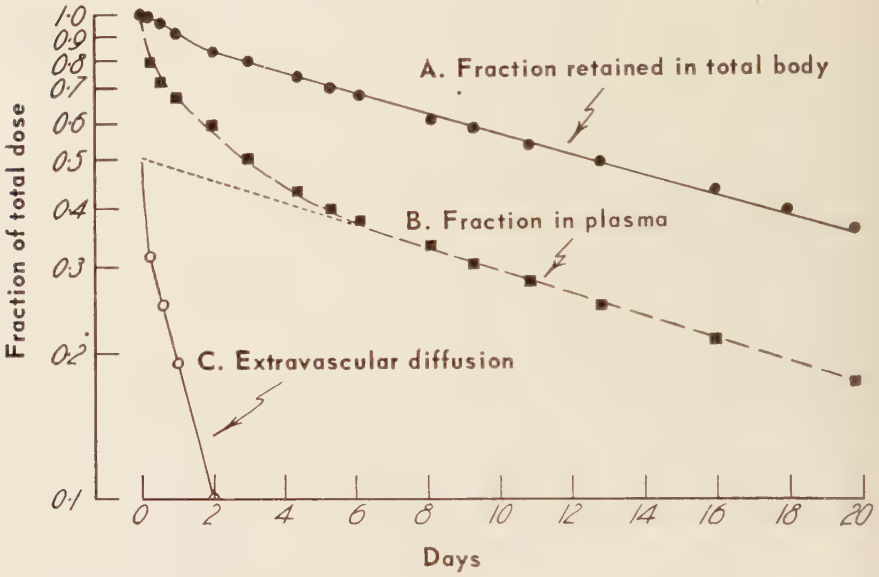

FIGURE 1. The disappearance of radioiodinated human serum albumin in a normal child: (A) from total body as estimated from urinary excretion data alone; (B) from the plasma; and (C) estimated extravascular migration. The broken line between (B) and zero time represents the extrapolation of the catabolism phase.

all of the tissues of the body, as well.[6] After the intravenous infusion of γ-globulin, the fall in the concentration of this protein in the plasma follows a pattern like that already described. Within 24 hours after the infusion, γ-globulin is readily detected in the connective tissues by the use of the fluorescent antibody method. Similarly, in the case of children with congenital afibrinogenemia, the intravenous infusion of fibrinogen results in the rapid appearance of fibrinogen in the connective tissues from which it was absent previously[7] (FIGURE 2).

There is little doubt today that the numerous and greatly varied species of proteins that characterize plasma are, in fact, to be found in all interstitial fluids and, in some cases at least, intracellularly as well. The use of the fluorescent antibody method of Coons[8-10] has demonstrated, for example, that plasma albumin, γ globulin, iron-binding globulin, β-lipoproteins, and fibrinogen are found in all connective tissues and interstitial spaces, as well as in the lymphatics and blood vessels and in many cells, particularly in

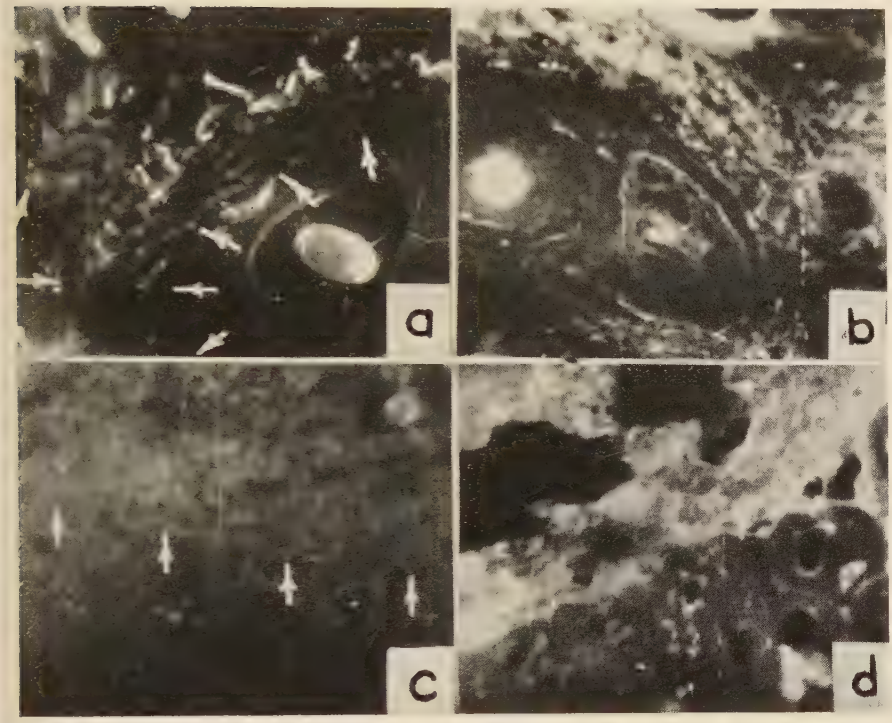

FIGURE 2 Skin (a and b) and muscle biopsies (c and d), stained for fibrinogen (white areas) by the fluorescent antibody method, before (a and c) and after (b and d) the infusion of fibrinogen in a child with congenital afibrinogenemia.

the cell nuclei[6] (FIGURE 3). Moreover, the cellular distribution of the plasma proteins studied is not the same for each plasma protein. Thus, while albumin may be present in the cells of a number of different tissues or organs, fibrinogen is not found to any great extent in any cells. Everything considered, however, the amount of a given plasma protein that is present intracellularly seems relatively insignificant compared to the amounts seen in the interstitial fluids and in the plasma.

It is quite apparent that the large quantity of plasma protein present in the extravascular-extracellular spaces is not a stagnant pool. That this protein re-enters the vascular system is readily demonstrated.[11] For example, in certain newborn children with Rh incompatibility, the plasma contains free anti-Rh antibodies. Since the antibodies are transferred from the mother to the fetus before the birth of the baby, one should anticipate the presence of anti-Rh antibodies in the extravascular fluids of the newborn child. During exchange transfusion with normal blood, the concentration of these antibodies in the plasma falls rapidly. Soon after the exchange ends, however, when about 85 per cent of the child's blood has been replaced in about an hour's time, the concentration of anti-Rh antibodies in the plasma rapidly rises again, although never to the level present before the exchange.

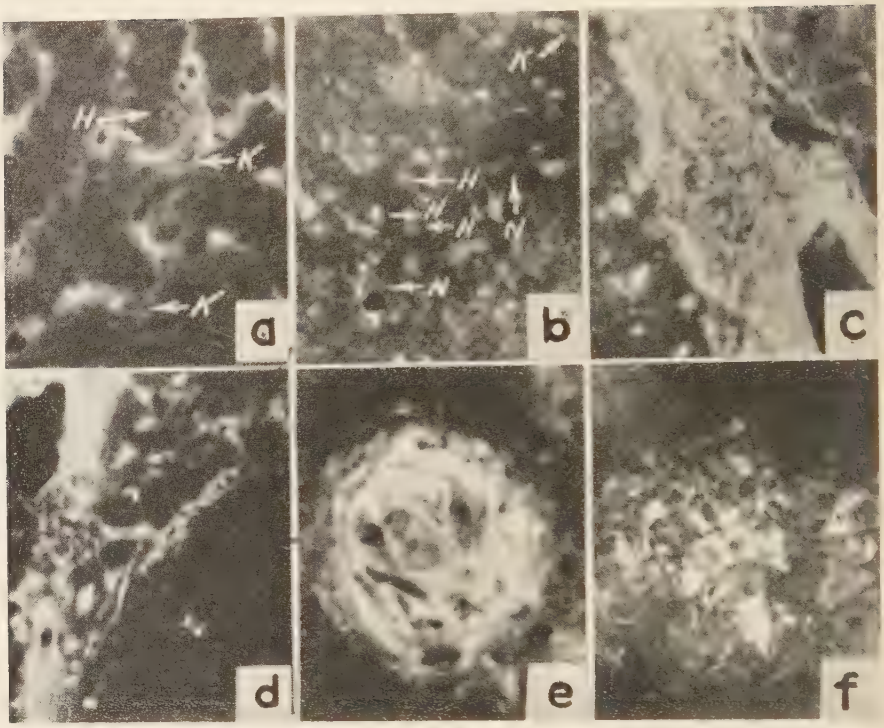

FIGURE 3. Human tissues sectioned at 4 μ and stained for specific plasma proteins by the fluorescent antibody method:

(a) Liver stained for γ-globulin. H = hepatic nuclei; K = Kupffer cells containing γ-globulin in their cytoplasm.

(b) Liver stained for albumin after most of the albumin was removed from the sinusoids. H = hepatic nuclei; K = Kupffer cell; N = endothelial cell.

(c) Muscle biopsy with γ-globulin in connective tissue.

(d) Muscle biopsy showing fibrinogen in connective tissue.

(e) Thymus showing γ-globulin in Hassall's body.

(f) Antidiphtheria antibodies in the plasma cells of a stimulated lymph node.

This demonstration of the migration of extravascular protein to the vascular system can be duplicated easily in animals. Thus, rabbit antipneumococcus antibodies can be passively transferred to normal nonimmune rabbits. After the steady state is reached, the removal of intravascular antibodies by exchange transfusion is, of course, accompanied by a fall in the plasma concentration of the antibodies. This is followed, however, by a rise in the concentration of antibodies in the plasma; the antibodies then resume the phase of logarithmic decline (FIGURE 4).

This cycle—from the vascular system to the interstitial fluids and back to the vascular system—is what is meant by the dynamic equilibrium between the extravascular and the intravascular plasma proteins. The extravascular pool appears to represent a major portion of the protein reserve described by Whipple[6] but, unlike his concept, the pool is not intracellular

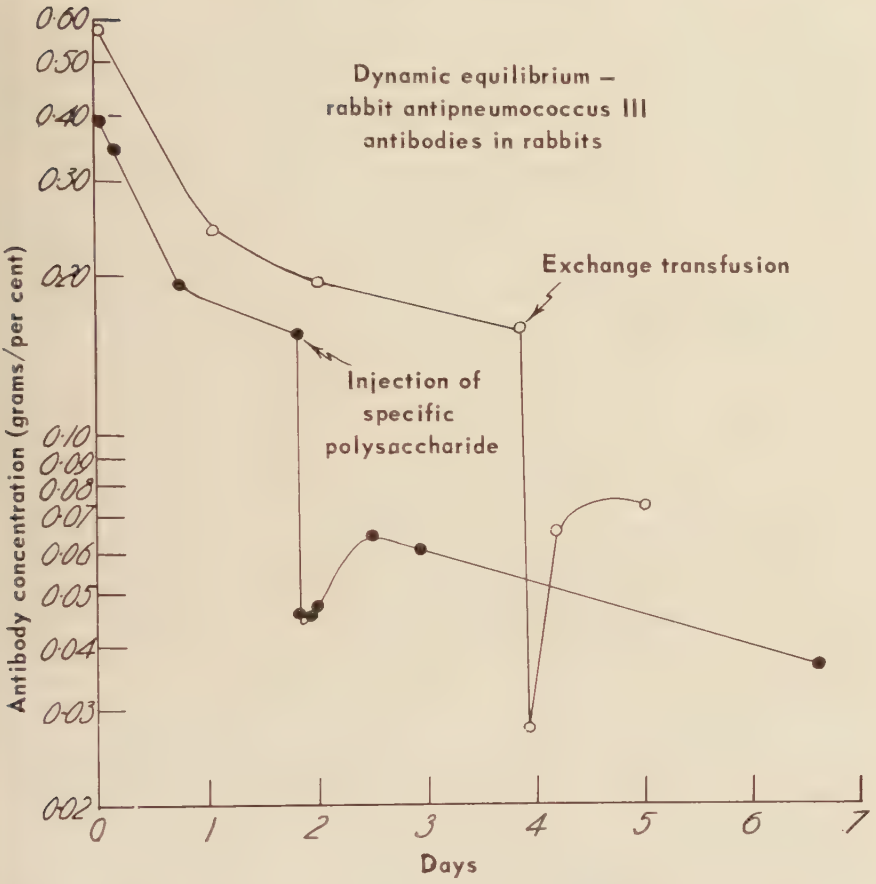

FIGURE 4. The disappearance of rabbit antibodies from the vascular system after passive transfer and the shift from the "interstitial fluids" to the vascular compartment after acute depletion of the latter.

and is already preformed. From considerations of the plasma disappearance curves of specific radioiodinated plasma proteins, and from analyses of other types of data, it has been found that roughly half of the total body pool of at least most plasma proteins normally is present extravascularly. This has already been stressed repeatedly in this monograph, so I shall not belabor the point.

Concentration of specific proteins in interstitial fluid under various conditions. As one would expect, the concentration of a given plasma protein in interstitial fluid is less than that in the plasma. The actual concentration in interstitial fluid can be obtained in certain tissues through the simultaneous use of immunochemistry and radiochemistry.[12] Thus, children and adults were injected intravenously with radioiodinated human serum albumin and, about 15 minutes later, skin and muscle biopsies were performed.[13] At the same time that the tissue samples were taken, venous

blood was drawn and the serum separated from it. The tissue samples were homogenized in buffer to a definite volume, V_m. The concentrations of radioactivity in the serum and tissue homogenates, R_s and R_m, respectively, were estimated, as were the concentrations of albumin in the serum, A_s, and the homogenate, A_m. The amount of albumin, A_v, contributed to the tissue sample by serum trapped in the vascular bed could be calculated:

$$\frac{V_m R_m}{R_s} = \frac{A_v}{A_s} \quad \text{or} \quad A_v = \frac{V_m R_m}{R_s} A_s \tag{1}$$

The total amount of serum albumin in the tissue sample, $V_m A_m$, less the vascular serum albumin, A_v, was the amount of albumin, A_e, present outside the vascular system:

$$\begin{aligned} A_e &= V_m A_m - A_v \text{ or} \\ A_e &= V_m\left(A_m - \frac{R_m}{R_s} A_s\right) \end{aligned} \tag{2}$$

By using chloride space as an estimate for interstitial space, the concentration of albumin in the interstitial fluid of skin and muscle in normal human beings was found to be about 0.7 to 0.9 gm. per cent. Interestingly enough, the amount of extravascular albumin found in the tissue samples was approximately equal to the amount in the vascular bed of the tissue samples.[12, 13]

This same type of determination has been done in a variety of ways[14] and for a number of different plasma proteins. However, more important at the moment is the fact that the concentration of a specific plasma protein in lymphatic fluid appears to be representative of the interstitial fluid that it drains.[12] Interstitial fluid protein apparently does *not* become more concentrated as the fluid passes along the lymphatics, since interstitial fluid and lymphatic fluid seem to be alike in protein composition and concentration. It would seem, therefore, that the net removal of protein from the extravascular pool is by way of the lymphatics, and that it is thus dependent upon the bulk flow of fluid rather than upon the diffusion of plasma protein.

On the other hand, the net transfer of protein to interstitial fluid is by way of the capillaries. Consequently, the concentration of protein in interstitial fluid is dependent upon the relative net transfer of water and protein from the capillaries, and thus it is also dependent upon the rate of lymphatic flow. Normally, there is a net transfer of water from the capillaries to interstitial fluid at the arteriolar end of the capillary due to hydrostatic pressure and a net transfer to the capillaries at the venular end as the result of oncotic pressure. The over all result is a net transfer of water from the capillaries to the interstitial fluid, and this is removed by way of the lymphatics by bulk flow. The diffusion of protein from the capillaries, however, is toward the interstitial fluid along the entire length of the capillary; at no time does the concentration of protein in the interstitial fluid normally exceed that in the capillary, and the concentration gradient remains toward the interstitial fluid.

It is easily seen, therefore, what results might follow after alterations of the hemodynamics that govern transfer of water and protein across the capillary wall. Consider, for example, a loss of oncotic pressure without alteration of capillary permeability to protein, such as occurs in nephrosis. Theoretically, this would cause a greater net transfer of water from the capillary to the interstitial fluid. Consequently, the result should be: (1) a concentration of protein in the interstitial fluid relative to that in the plasma lower than that in the normal individual, and (2) an increased lymphatic flow. This expectation has proved to be justified.[15, 16]

If, in another type of case, capillary permeability to protein is increased, as in instances of increased venous pressure, the situation is more complex, since the concentration of protein will depend upon the degree of increase in capillary permeability as well as the net transfer of water. Where venous pressure is increased, the net transfer of water obviously will be greater than normal, and lymphatic flow will be increased. Thus, with a moderate increase in venous pressure, the *concentration* of protein in interstitial fluid need not exceed and may even be lower than normal, although the *amount* of protein transferred by the lymphatics will be greater than normal. With additional damage to the capillary due to hypoxia or inflammation, the concentration of protein in the extravascular fluid may easily exceed the normal, due to a greater increase in capillary permeability to protein.

Finally, if the lymphatic flow is blocked, or stopped, or is not available, as in lymphedema or in certain cysts, the concentration of a plasma protein eventually will reach levels comparable with those in the plasma.[17] The net transfer of water will become minimal, approaching zero, depending upon the degree of cessation of lymphatic flow, while the net transfer of protein will continue until the concentration gradient is eliminated.

Returning to the "simple" plasma disappearance curve, then, this curve is by no means so simple as it appears. The curve reflects the net deficit to the vascular system due to dynamic equilibrium and loss due to catabolism or degradation. Consequently, it is almost superfluous to remind the reader that, during the "steady state," the diffusion of protein from the vascular system is matched by the return of protein by way of the lymphatics, the steady logarithmic decline being due to the increment of loss to the system due to catabolism. In such a system, then, *at no time* will uniform specific activities exist throughout the system.

Catabolism of Specific Plasma Proteins as a First-Order Reaction

Before we discuss the effect of such nonuniformity of specific activity on our interpretation of data on radioiodinated plasma protein, let us examine more closely the catabolism portion of the protein disappearance curve. It is quite apparent that, in any given individual in the steady state, a given fraction of the total body pool of a given plasma protein, including the labeled protein, is catabolized per unit of time, and that the catabolized fraction is replaced simultaneously by unlabeled plasma protein. Thus, the catabolism curve must assume the form of a single exponential when the amount of label in the system is plotted against time. If the data obtained by radio-

iodinated tracers is to be interpreted properly in pathological conditions, however, it becomes necessary to know whether or not catabolism is actually a first-order reaction. For example, consider the situation in which there is a severe reduction in the body pool of a particular plasma protein. Under these circumstances, does the body demand the catabolism of a certain minimum amount of that plasma protein, or is catabolism a first-order reaction? Let us say, for the sake of discussion, that the body pool had been reduced to one-tenth of its normal level, and that the normal rate of catabolism was x gm./day. Then, in other words, will the rate of catabolism remain at or close to x gm./day, or will it fall to $x/10$ gm./day?

The available evidence at the moment appears to suggest the concept that plasma protein catabolism may be a first-order reaction:

(1) Severe reduction of the total body pool of plasma protein in dogs by plasmapheresis did not increase the fractional rate of catabolism of labeled homologous plasma albumin.[18, 19]

(2) In children with congenital agammaglobulinemia, injected γ-globulin had the same fractional rate of catabolism, whether the plasma levels of γ-globulin were 600 to 800 mg. per cent or less than 50 mg. per cent.[20] Similarly, in children with congenital afibrinogenemia, the fractional rate of catabolism of injected fibrinogen remained the same from plasma levels of fibrinogen of over 100 mg. per cent to levels approaching zero.[7]

(3) In children with severe protein malnutrition resulting in decreased plasma albumin synthesis and consequent reduction of the body pool of albumin, the fractional rate of catabolism of albumin was within relatively normal limits.[21] It must be noted, however, that in these instances there was no apparent change induced in the permeability of the renal glomerulus to plasma protein. Hughes[22] recently has indicated that a major part of albumin catabolism in mice might be due to reabsorption and degradation of the protein in the renal tubules. Under these circumstances the tubules would degrade whatever albumin was presented to them up to a certain maximum amount per unit of time, and any albumin in excess of this would be excreted in the urine. Since the glomerular filtration of albumin would be analogous to a first order reaction, then a major limiting factor to degradation would be the permeability of the glomerulus or the glomerular filtration of albumin and, consequently, of the first order. Of the fraction of albumin catabolized elsewhere in the body, the data would suggest this to be of the first order as well. In the presence of intact glomerular permeability, then, certainly the limiting factors to catabolism appear to be of the first order, whether a given limiting factor is the permeability of cell membranes (in the liver, for example) the glomerular membrane, or both.

Some Considerations of Calculations of Plasma Protein Turnover

Significance of measurements of volume of distribution. To calculate the actual rates of catabolism, or synthesis, of a given plasma protein, it is necessary to know the size of the body pool of that protein. As of now, this is most conveniently calculated from the concentration of the protein in the plasma and its volume of distribution. The concentration of a plasma protein

frequently can be estimated reasonably well by a variety of methods, but the volume of distribution is not determined so easily. The volume of distribution frequently has been estimated by extrapolation of the phase of logarithmic decline of a plasma disappearance curve to zero time[3] (FIGURE 1). As has been pointed out by Berson and his colleagues,[4] this extrapolation, due to possible variations in the amount of labeled protein catabolized per unit of time prior to the establishment of the steady state, is not quite correct as a straight line. In addition, the nonuniformity of specific activities in the various compartments assumes significance.[5, 23] Thus, the volume of distribution determined by this method will be in error to a degree reflected not only by the extent that the extrapolated straight line deviates from the true catabolism curve, but also to the extent that the specific activities present extravascularly differ from those in the plasma.[24] Berson has suggested the use of data obtained from the urinary excretion of degradation products to correct the volume of distribution for the possible variations in catabolism. This has proved entirely adequate under most circumstances, but this method, too, has its difficulties, since it requires that: (1) the excretion of degradation products in the urine be prompt, and (2) the specific activity of the radioiodoprotein be the same in all compartments. Even under the best conditions, neither of these conditions is met exactly. Normally, the fractional rate of excretion of tagged degradation products and the fractional rate of extravascular diffusion of iodoprotein are relatively rapid compared to the fractional rate of catabolism and, consequently, the two conditions are almost fulfilled. Both methods have proved useful in many situations, since exact measures of the total body pool for a given plasma protein have little exact meaning at this time.

However, under certain circumstances the volume of distribution of a given plasma protein cannot be measured at all closely by these methods. The faster the fractional rate of catabolism and/or the slower the relative fractional rate of excretion of degradation products, the greater will be the

TABLE 1

THE TURNOVER OF ALBUMIN IN CHILDREN WITH THE NEPHROTIC SYNDROME

Patient	State of disease	Approximate half time of turnover			
		From plasma disappearance (days)	Total* (days)	Urinary loss* (days)	Catabolism* (days)
R. S. B.	Latent	12.0	14.7	98.8	17.5
A. B.	Mild	2.3	2.0	7.5	2.8
E. T.	Mild	2.0	2.2	2.9	8.7
K. S.	Full-blown	1.8	1.1	3.3	1.7
S. L.	Full-blown	2.1	0.8	1.4	2.1
D. W.	Full-blown	2.0	0.8	3.1	1.1

*From equations in text.

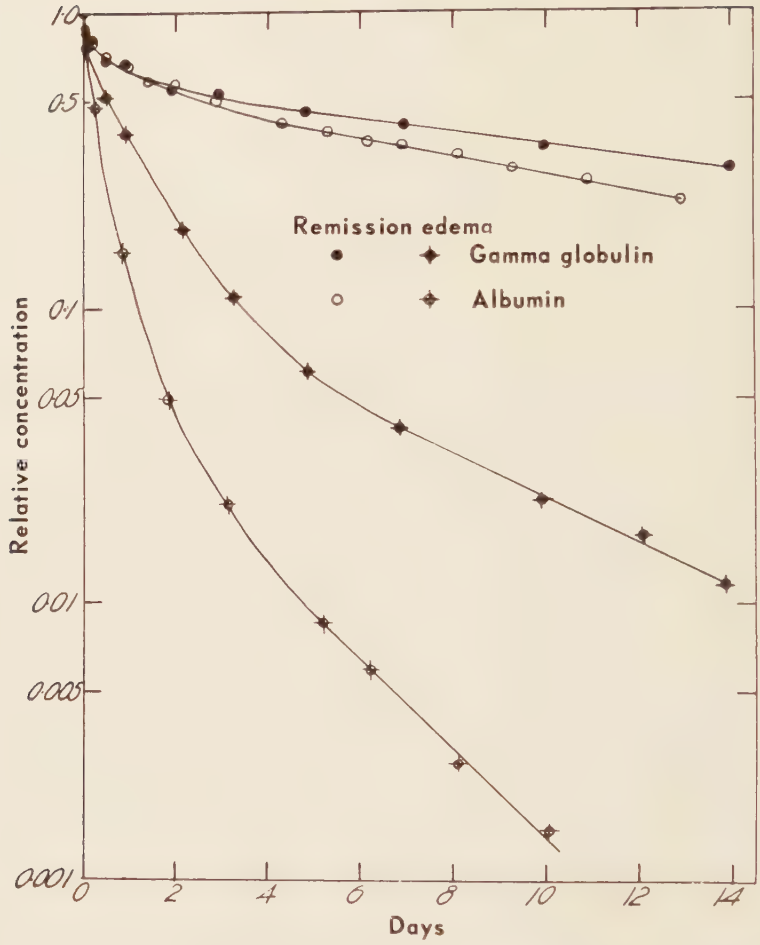

FIGURE 5. Plasma disappearance curves of albumin and γ-globulin in children with full-blown nephrotic syndrome and in remission.

discrepancy between true and estimated volumes of distribution. Thus, in the case of nephrosis, where there is a very high fractional rate of loss of iodoalbumin from the plasma, the result is a much higher specific activity in the interstitial fluid than in the plasma.[5] The consequence is a tendency toward a marked overestimation of the volume of distribution and an underestimation of the fractional rate of loss of iodoalbumin from the plasma due to catabolism and excretion (FIGURE 5, TABLE 1).

Proteinuria. However, despite all of these complications, it is possible for the picture to become even more confusing. Once having succeeded in estimating the volume of distribution of a given protein, and from this the total body pool thereof, let us consider the influence of an additional factor on our calculations, namely, proteinuria. It is obvious that this condition

will not only aggravate the nonuniformity of specific activity of radioiodoprotein in the various body fluids, but also makes interpretation of the slow component of the plasma disappearance curve very interesting. The slow component now has elements of at least two factors: catabolism and urinary loss. Also, if the proteinuria is severe and the fractional rate of catabolism is rapid, the slow component may show the effects of still a third element; that is, the result of a high specific activity in the extravascular fluids that re-enter the plasma. Thus, in a study of the nephrotic syndrome in children, where this situation was encountered with radioiodinated albumin, γ-globulin, and iron-binding globulin, another approach to the calculation of rates of catabolism was adopted.[5] Let us consider albumin as an example.

It can be shown that the total albumin synthesized per unit of time, A_s, can be obtained as follows:

$$A_s = \frac{A_e}{A^*/A_s^*} \tag{3}$$

where A_e is the amount of endogenous albumin lost in the urine per unit of time, A_s^* is the total dose of radioiodinated albumin, and A^* is the amount of radioiodinated albumin lost in the urine after an infinite time period. The amount of albumin catabolized per unit of time, A_c, was then calculated:

$$A_c = A_s - A_e \tag{4}$$

assuming, of course, that the loss of albumin from the body is due either to catabolism or urinary excretion (TABLE 1).

Metabolism of Low Density Plasma Lipoprotein: An Example of Conversion

In all of the discussions that have appeared thus far, it has been assumed that, during the peregrinations of an iodoprotein through the body (1) the iodoprotein started with remains unchanged until catabolized, and (2) the radioiodine is not transferred from one plasma protein molecule to another. Imagine the situation in which the difficulties discussed earlier regarding interpretation of iodoprotein data were compounded by the iodoprotein molecule not remaining the same during the period of study. Such is the situation with the low density lipoproteins, at least those of S_f 10 to 100.[25]

It was found possible to label the peptide moieties of low and high density β-lipoproteins with I^{131} and to study the metabolism of these proteins in normal individuals and in children with the nephrotic syndrome. It would appear from the data obtained that the lipoproteins of lower density, S_f 10 to 100, are normally converted to lipoproteins of higher density, S_f 3 to 8, at a very rapid rate. In the nephrotic child, this conversion takes place much more slowly and is accompanied by an increase in the rate of synthesis of the low density lipoproteins. The rate of catabolism of the high density lipoproteins is relatively normal in nephrosis. The over-all result is an elevated plasma level and body pool of the lipoproteins of low density, which result in the hyperlipemia and hypercholesterolemia seen in this disease

(FIGURE 6). It is interesting to note that the peptide moiety of α lipoprotein does not share in the metabolism of the β-lipoproteins, as has been suggested by others.[26]

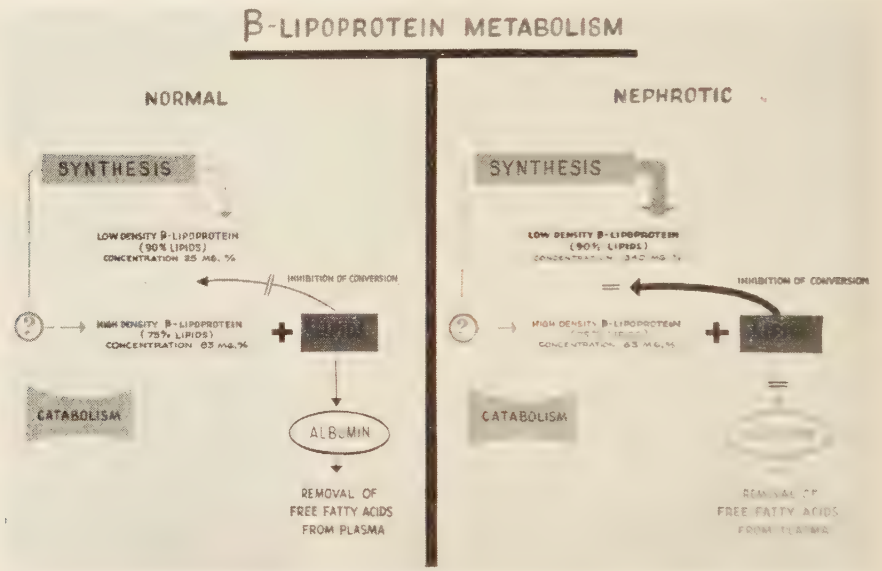

FIGURE 6. Simplified diagram of the metabolism of β-lipoproteins in normal and nephrotic children.

Summary

(1) An attempt has been made to discuss, albeit briefly, the dynamic equilibrium that exists between intravascular and extravascular plasma proteins; that is, the cycle from the blood plasma to the interstitial fluid by way of the capillaries and back to the vascular system by way of the lymphatics. The distribution of the plasma proteins in the body is described, as are the factors that govern the concentrations of these proteins in the interstitial fluids.

(2) From the evidence, it appears that catabolism of a given plasma protein is a first-order reaction; that is, that the fractional rate of catabolism is independent of the size of the body pool of that protein, while the rate of catabolism is a function of the size of the body pool.

(3) Some of the considerations that enter into calculations of plasma protein turnover have been discussed, and some of the sources of error have been emphasized, including the nonuniformity of specific activity of the iodoprotein in the various body fluids under different conditions. As examples of additional complications, the problem of proteinuria in relation to turnover calculations and the conversion of one iodoprotein into another are presented.

Acknowledgments

The experiments presented in this report and their interpretation were made possible through the cooperation, suggestions, and advice of many collaborators and colleagues. Among these were: Charles A. Janeway, Children's Medical Center and Harvard University Medical School, Boston, Mass.; Walter L. Hughes and James Robertson, Brookhaven National Laboratory, Upton, N. Y.; Charles Lewallen, National Institutes of Health, Bethesda, Md.; and John L. Oncley and Albert H. Coons, Harvard University Medical School, Boston, Mass.

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Discussion of the Paper

A. S. McFarlane (*The National Institute for Medical Research, London, England*): I should like to comment on Gitlin's references to nephrotic patients and to his recent important publication on this subject with Janeway and Farr.[1] The method used by those investigators to assess the ratio of protein metabolized to protein excreted daily in the urine by integrating total urinary diffusible and protein-bound activities over the full period of excretion and dividing one by the other has the great merit that it is independent of redistribution of labeled molecules between the plasma and the lymph. However, this method requires that the patient should be excreting a constant amount of protein daily, a situation that frequently is not found. It appears that the same information can be derived, over short periods, from measurements of plasma specific activities and urinary data in a patient not so stabilized with respect to proteinuria. Therefore I do not altogether share what seems to be a somewhat pessimistic outlook on the possibility of interpreting the condition of the patient from short-term measurements with I^{131} albumin.

Any consideration of the nephrotic patient that is based on plasma protein-specific activities, however, must take into account the possibility of renal selection of injected labeled molecules. In collaborative studies with colleagues at St. Mary's Hospital, London, England, we have found this to be the case in some nephrotic patients, in whom the half life of plasma albumin declined more slowly than did that of urinary albumin. On the other hand, albumin or total protein prepared from the urine and labeled with I^{131} on reinjection into patients proved to be completely free of this effect: that is, plasma and urinary protein-specific activity curves remained parallel throughout. We can recommend this material as ideal tagged protein for studies of nephrosis.

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THE CONTRIBUTION OF I^{131}-LABELED PROTEINS TO MEASUREMENTS OF BLOOD VOLUME

By E. B. Reeve

Department of Medicine, University of Colorado School of Medicine, Denver, Colo.

All measurements of intravascular volume depend on the simple mathematical relationship $V = N/n$. A known quantity, N, of a substance is introduced into the circulation and, when it is distributed uniformly through the circulating blood, samples of blood are withdrawn, and the quantity of dissolved substance n in 1 ml. is determined. The volume in question, V in ml., is then calculated from $V = N/n$. For a measurement of plasma volume, the ideal substance would be readily measurable in high dilution, and after injection it would leave the circulation very slowly or not at all, except by hemorrhage. Such a substance is not yet known, but some proteins, particularly "labeled" proteins, and a few polysaccharides of high molecular weight approximate these requirements. These substances leave the circulation slowly by leaking into the extravascular spaces and, after an interval, slowly return to the circulation by way of the lymphatic ducts.[1-3] To obtain a fair and meaningful measurement of n, correction must be made for this leakage. This is done by measuring the fall in concentration of the injected substance in a series of blood samples withdrawn after a short interval has been allowed for mixing in the circulation, and correcting for the loss by extrapolation to the time of injection.

Because of the great amount of careful work that has been done with it, the reference substance for measurements of plasma volume is the dye T-1824. Fifteen years ago[4] it was shown that T-1824 in the quantities given for plasma volume measurements is bound firmly to plasma albumin. Evidence later was provided[5] that the reaction between T-1824 and plasma albumin occurs rapidly *in vivo*, and that in a model system consisting of bovine albumin and T-1824 in the concentrations found in animals in which the plasma volume is being measured, about 999 out of every 1000 molecules of dye are bound by the albumin.[6] Thus, T-1824 can be regarded as a plasma-albumin label that reacts rapidly with, and is almost completely bound by, plasma albumin when injected in the circulation.

A number of workers have questioned the validity of measurements of plasma volume with T-1824. Examining the equation $V = N/n$, clearly the only measurement that can be influenced by vital processes is that of n, the concentration of T-1824 in the plasma. We can conceive only of processes that remove T-1824, and these would result in a low value of n and an overestimate of plasma volume. A slow regular rate of removal of dye should not matter, because it would be corrected for by the method of extrapolation. However, the kind of loss that would be difficult to detect, or correct for, is what has been called the "gobble."[7] This pictures a portion of the dye as being removed shortly after its injection and during the period of mixing in the blood stream, either before it has become bound to the protein

or by being pulled from it. The latter conjecture is not impossible, for it has been shown that serum albumin from one species can displace T-1824 from combination with serum albumin of another species.[8] So far, although the "gobble" often has been suggested, it has never been demonstrated conclusively, and strong evidence has been brought against it.

Many experiments have been made to test the validity of the T-1824 measurements of plasma volume. Table 1 summarizes results from 2 groups

Table 1

Comparison of T-1824 Measurements of Plasma Volume in Dogs: (1) with Antigen[9] Measurements, and (2) with Hemoglobin[10] Measurements

	Number of measurements	Mean difference (per cent)*	Standard deviation of differences
(1) *Antigen*			
Bovine albumin	10	+1	±4.0
Polysaccharide SIII	4	−2	±4.1
(2) *Hemoglobin*			
Dog hemoglobin	10	−0.7	±3.2

*T-1824 taken as 100 per cent.

of experiments[9, 10] made on dogs, in which the volume of distribution of T-1824 in the blood stream was compared with the volume of distribution of 3 substances of high molecular weight. In the first group of experiments it is seen that there is very close agreement between the plasma volume measured by T-1824 and that measured by the antigens, bovine albumin, and the polysaccharide SIII. In the second group of experiments it is seen that the plasma volume measured by T-1824 agrees closely with that measured by the animal's own hemoglobin solution injected intravenously. These injected substances had quite widely differing loss rates from that of the plasma, and the agreement of all these measurements strongly supports the idea that T-1824 measures the true plasma volume in the dog. However, these substances are not suitable for use in man, and such experiments have not been reported for other animals.

In the last 15 years a number of methods for measuring the circulating red cell volume have been devised or improved.[11-16] Formerly, an estimate of the circulating red cell volume often was attempted from the measured plasma volume and the measured percentage of cells in venous blood (that is, the venous hematocrit corrected for plasma trapped in the cell column), from the equation:

$$\text{Circulating red cell volume} = \text{measured plasma volume} \times \frac{\text{percentage of venous cells}}{\text{percentage of venous plasma}}$$

It was natural, therefore, to compare estimates made by this method, using T-1824 to measure plasma volume, with direct measurements made by various labeled cell methods. Table 2 summarizes the results of some of

TABLE 2

T-1824-HEMATOCRIT ESTIMATES OF RED CELL VOLUME COMPARED WITH P^{32}-LABELED CELL MEASUREMENTS

	Percentage of range of venous cells	T-1824-Hematocrit estimate*	P^{32} Measurement
Man[17]	40–50	116–120%	100%
Dog (splenectomized)[18]	40–50	118–122%	100%
Rabbit[19]	30–40	125–130%	100%

*The T-1824-hematocrit estimates are calculated as a percentage of the P^{32} measurement.

these measurements in man, the splenectomized dog, and the rabbit, and it shows that the estimate of cell volume given by the T-1824 hematocrit method is some 15 to 30 per cent higher than that given by the P^{32}-labeled cell method. The P^{32} method has been shown to give results in close agreement with those obtained with Fe^{55}-labeled cells in the dog[20] and Cr^{51}-labeled cells and cells labeled by a blood-group difference in man,[13, 21, 22] so that we have reason to accept all these labeled-cell methods as measuring the same true red cell volume. Accepting this, there are 2 possible explanations of the differences shown in TABLE 2: either T-1824 gives an overestimate of plasma volume, or the assumptions made in estimating the cell volume from the plasma volume and the hematocrit are incorrect. The experiments already described in the dog would lead us to believe that the T-1824 measurements of plasma volume are correct in this animal, and to question the assumptions used in estimating the cell volume from the plasma volume and the hematocrit.

To obtain a correct estimate of the cell volume from the plasma volume and the hematocrit, the percentage of cells in the venous blood must represent the average distribution of cells in the blood of the whole body. This may be shown as follows:

Let $H_{av.}$ = average percentage of cells in the whole body, or (cell volume × 100)/(cell volume + plasma volume). Let Hct = the percentage of cells in the venous blood as determined from the venous hematocrit corrected for trapped plasma. Since the plasma volume-hematocrit estimate of cell volume = plasma volume × $Hct/(100 - Hct)$ and the true measurement by definition = plasma volume × $H_{av.}/(100 - H_{av.})$, then if the plasma volume estimate of cell volume is to be true, $H_{av.}$ must equal Hct, or $H_{av.}/Hct$ must equal 1.0. This ratio $H_{av.}/Hct$ has been termed F_{cells} or, more widely but with a rather doubtful connotation, "body hematocrit"/venous hematocrit. The best demonstration that Hct, the venous hematocrit, can vary quite independently of $H_{av.}$, the average distribution of cells in the animal's total blood, is seen in the dog with an intact spleen.[18] In this animal the hematocrit level fluctuates widely, depending on the state of filling of the spleen with blood rich in cells and poor in plasma. However, measurements of total plasma volume and total cell volume during these fluctuations

show little change, and hence an almost stationary value of $H_{av.}$, the average proportion of cells in the total blood. As a consequence, the ratio $H_{av.}/Hct$ can vary from about 1.10 in the dog under pentobarbital sodium (Nembutal) with its spleen engorged with red cells to about 0.90 in the dog with its spleen emptied by an injection of adrenaline. Clearly it is only by chance, when the spleen of the dog is partly filled, that $H_{av.} = Hct$. However, the dog with an intact spleen and varying values for the ratio $H_{av.}/Hct$ is quite unlike the dog without its spleen, and man and the rabbit as well. Table 3 shows some representative values for the ratio F_{cells} in man, the

Table 3

The Ratio F_{cells}, $\frac{\text{"Body Hematocrit"}}{\text{Venous Hematocrit}}$, as Determined from P^{32}-Labeled Cell Measurements of Red Cell Volume, T-1824 Measurements of Plasma Volume and the Percentage of Cells in Venous Blood

	Number of observations	Venous cell range (per cent)	F_{cells}	Standard deviation
Man[17]	28	8.7–77.6	0.910	±0.026
Splenectomized dog[18]	16	36.9–50.5	0.899	±0.023
Rabbit[19]	29	29.2–42.6	0.85*	±0.034

*See below for a revised value.

splenectomized dog, and the rabbit. Note that in all three this ratio is below 1.0, and that in man and the splenectomized dog it is remarkably constant. Thus, Chaplin, Mollison, and Vetter[17] found in men with hematocrit levels ranging from 9.1 per cent to 82.3 per cent an average value of 0.91, with a standard deviation (S.D.) of 0.026; in resting splenectomized dogs the ratio is 0.899 ± 0.023. In rabbits the reported ratio is somewhat lower, averaging 0.85 with S.D. of 0.034; this indicates more scatter in the values. The values below 1.0 show that, if the measurements of cell volume and plasma volume are correct, $H_{av.}$, the average percentage of cells in the total blood, is lower than Hct, the percentage in the venous blood. This implies that, at some sites in the animal body, there is blood with relatively fewer cells than are present in the venous blood. A considerable amount of independent evidence shows this to be the case, and that the blood in the small vessels of less than 0.2 mm. in diameter contains relatively fewer cells than the blood in the larger vessels.[23-26] There is also independent evidence that the blood in tissues and organs contains proportionately fewer cells than does the venous blood.[27-29]

We may summarize the experiments so far reviewed by saying that there is good evidence to show that T-1824 measures the true plasma volume in the dog. There is less evidence to indicate that this dye measures the true plasma volume in man and the rabbit; however, if the distribution of cells and plasma in the splenectomized dog is comparable to that in man, who has a relatively small and inactive spleen, then the close similarity between

the F_{cells} ratio in man and the splenectomized dog is additional evidence. Furthermore, it is very difficult to explain the constancy of the F_{cells} ratio in the study of Chaplin, Mollison, and Vetter[17] in patients with widely varying hematocrit levels as being due to T-1824 "gobbling." Clearly, however, it would be most valuable, especially in man and in animals other than the dog, to have additional satisfactory substances available for measuring plasma volume. The advent of the I^{131}-labeled proteins, with their ease and accuracy of measurement, has provided us with such substances.

The I^{131}-labeled protein that has chiefly been used for the measurement of plasma volume is serum albumin. Before discussing the results obtained with this material, the problem of what sort of preparation might be expected to give reliable results should be considered. In general, one would anticipate that the best measurements would be obtained if at least 99 per cent of the radioactive label were firmly bound to the protein, if the protein were minimally altered by the iodination process, and if the albumin labeled were from the same species of animal as that in which the measurement was made. The proportion of the total radioactivity that is easily split off is measured best by precipitating the labeled protein with cold trichloroacetic acid and determining what proportion of the total counts is in the supernatant. If any significant proportion of the total counts is found there, this fact will give rise to errors, depending for their extent on the rate of removal of this loosely bound label from the circulation. If a portion of the protein is denatured by the process of iodination or by the dose of radiation to which it is exposed, and if this portion is removed rapidly from the circulation after injection, further errors will arise. Finally, if I^{131} albumin from one species is repeatedly injected into another species, then if the recipient develops an immunity reaction to the foreign protein, rapid removal, with consequent errors, may result. It may be noted that few reports give sufficient information to enable us to determine how closely the preparations used meet all of the above criteria.

Since T-1824 is thought to label the lysine groups of albumin, and since iodine labels primarily the tyrosine groups,[30] it is of interest to compare the intravascular behavior of T-1824-labeled and I^{131}-labeled albumin. Table 4 summarizes the quantities of these 2 labeled albumins lost from the blood stream 1 hour and 24 hours after injection in man, in the dog, and in the rabbit. Note that homologous albumin labeled with I^{131} was injected in man

Table 4

Percentage of Loss of T-1824 and I^{131} Albumin from the Plasma After Intravenous Injection

	After 1 hour		After 24 hours	
	T-1824%	I^{131}-albumin%	T-1824%	I^{131}-albumin%
Man[31–33]	11	11.5	49	40
Dog[34]*	10	9	72	47
Rabbit[19,35,36]	25	15	ca. 95	ca. 50

*I^{131}-labeled human albumin was used.

and the rabbit, but that human albumin labeled with I^{131} was used in the dog. It is seen that after 1 hour in man and the dog there is not much difference in the percentages of loss of the 2 materials, but that after 24 hours slightly more T-1824 than I^{131} has left the circulation of man and considerably more has disappeared from the circulation of the dog. In the rabbit, however, about 25 per cent of the T-1824 has left the circulation in the first hour, compared with about 15 per cent of the I^{131}; after 24 hours about 95 per cent of the T-1824, as compared with about 50 per cent of the I^{131}, has disappeared. This must mean that T-1824 is bound most strongly by human albumin and least strongly by rabbit albumin. This has been confirmed by the observation that human albumin can displace T-1824 from dog albumin fairly readily, but particularly readily from rabbit albumin.[5] These diverse observations suggest that I^{131}-labeled albumins are potentially at least as good as T-1824 albumin for measuring plasma volume, at least in man, the dog, and the rabbit.

The first measurements of plasma volume with I^{131}-labeled serum albumin and a comparison of these measurements with T-1824 measurements were reported by Gibson and his co-workers[37] in 1946; in the last 5 years, particularly since the availability of commercial preparations of I^{131}-labeled serum albumin, there has been a spate of measurements by this method. A number of comparisons between measurements of plasma volume made simultaneously with T-1824 and I^{131} albumin have been reported.[31, 34, 38-41] TABLE 5 summarizes the results of 2 careful studies made

TABLE 5

COMPARISON OF SIMULTANEOUS MEASUREMENTS OF PLASMA VOLUME BY T-1824 AND HUMAN I^{131} ALBUMIN

	Number of measurements	Mean difference*	Standard deviation of differences
Man[31]	28	+2%	±6.0
Dog[34]	13	−0.5%	±2.0

*T-1824 value taken as 100 per cent.

in man and in the dog. These 2 studies, which show good agreement between the results obtained by the 2 methods, have been chosen for a reason that will be discussed below. A few others[38] have reported fair agreement; however, others[40-42] have reported poor agreement, with T-1824 giving the higher measurement.

This was the situation when, about a year ago while we were studying the biological behavior of rabbit I^{131} albumin, it was noted accidentally that plasma volume measurements made with T-1824 were about 10 per cent higher than measurements derived from the I^{131}-albumin data. Since the most likely explanation seemed to be technical error, a series of tests was made to determine the question. These tests, of which a sample is given in TABLE 6, soon showed that serious errors could arise from adsorption

Table 6

Loss of I^{131} Albumin from Solutions in Volumetric Flasks by Adsorption[42]

	Solvents		
	Distilled water	0.9% NaCl	Carrier Protein in 0.9% NaCl
Counts per min. per ml.			
(1) After 90 min., at 25° C.	3190	3760	4250
	75%	88.5%	100%
(2) After 20 hr., at 4° C.	1960	3050	4350
	46%	72%	102.5%
(3) After adding NaOH	4160	4040	—
	98%	95%	

At zero time, 27 μg. of I^{131} albumin was added to pairs of 250-ml. volumetric flasks containing distilled water, 0.9 per cent NaCl, and 0.9 per cent NaCl with 1 ml. of human plasma per 100 ml. of saline. Samples were removed at the times shown. Immediately after the samples labeled (2) had been removed, 300 mg. of NaOH was added to the flasks, the contents of which were mixed by inversion, and then the samples labeled (3) were removed. Loss of counts from the water and saline solutions are demonstrated, particularly after refrigeration. Most of these reappear after adding NaOH.

losses of I^{131} albumin in water or 0.9 per cent NaCl to volumetric glassware. A number of observations on this adsorption have been made and are described elsewhere,[42] but the points relevant to measurements of plasma volume are these:

First, the proportion of the total protein adsorbed from solution depends on the surface area of the glass with which the solution comes in contact, and this depends on the surface-to-volume relationship of the glassware. For instance, syringes and pipettes with large surface-to-volume relationships potentially can remove much more protein from solution than can a large volumetric flask with a small surface-to-volume relationship.

Second, the concentration of protein is very important. Ordinarily, above a concentration of 0.5 mg. of protein per ml. in almost any piece of commonly used glassware, less than 1 per cent of the contained protein is adsorbed. For this reason, no significant losses will occur from plasma samples containing about 50 mg. of protein per ml. For a given area of glass, the greatest proportionate losses occur with concentrations of protein of 10 μg./ml. or less. The latter range of concentrations is that encountered in the dilutions of I^{131} albumin commonly made up as standards for the determination of N in the equation discussed earlier in this paper. It is also the range of concentrations transferred in pipettes and often injected. The net effect of these adsorption losses is to cause an underestimate of plasma volume that fairly easily reaches 10 per cent of the total and, under very unfavorable conditions, may reach 20 per cent.

It was noted above that the experiments of Schultz and his co-workers,[31] and Sear, Allen, and Gregersen[34] were chosen for a particular reason. In making their standards, both groups protected them against adsorption

losses, the first by diluting with human plasma, the second by diluting with a 1 per cent detergent solution that blocks adsorption. This procedure greatly reduces adsorption errors. However, neither group protected its syringes or pipettes against such losses. This is best done by making up the I^{131}-albumin solution for injection with sufficient homologous plasma to render adsorption of it insignificant. Without such protection, errors of as much as 5 per cent, and perhaps more, might still arise.

Franks and Zizza[43] therefore made another series of comparisons in man, taking precautions to prevent any adsorption errors. In a series of 12 patients and medical students these investigators found close agreement between the results obtained by the 2 methods, provided that the 2 to 6 per cent of the radioactivity of the commercial preparation of I^{131} albumin they have used that remains in the supernatant after precipitation by cold trichloroacetic acid is excluded from the calculation. In this case, the results average very slightly higher than the results obtained by the use of T-1824. If this residue is not excluded, the volumes are quite appreciably higher. This nonprecipitable I^{131} often seems to have been included in the calculations of other workers. The results of Franks and Zizza in man seem reassuring; from the work on the dog already discussed, we should expect that, when all adsorption losses are prevented in this animal, there will still be close agreement.

However, this is not true in the rabbit. Table 7 summarizes some recent results obtained by Zizza and myself[35] in simultaneous measurements made

Table 7

Simultaneous Measurements of Plasma Volume with T-1824 and I^{131} Albumin in Rabbits[35]

Number of measurements	Mean difference*	Standard deviation of differences
9	−8.2%	3.2

*T-1824 taken as 100 per cent.

with T-1824 and several preparations of rabbit I^{131} albumin, meeting the requirements of a good preparation specified earlier. It is seen that the I^{131}-albumin method gives about an 8 per cent lower value than T-1824, and the 10 per cent deviations noted earlier, therefore, were not due to absorption errors. If technical errors are avoided it is almost axiomatic that the lower of the 2 measurements, if significantly lower, is the more true, so that the present I^{131}-albumin measurements in rabbits may be taken as correct. Using these recent values for plasma volume, one may now calculate a new value for F_{cells}; this averages 0.89.

What is the reason for the difference in plasma volume measurements with T-1824 and I^{131} albumin in the rabbit? Is it some difference in the binding of T-1824 by rabbit albumin, as compared with human and dog albumin,

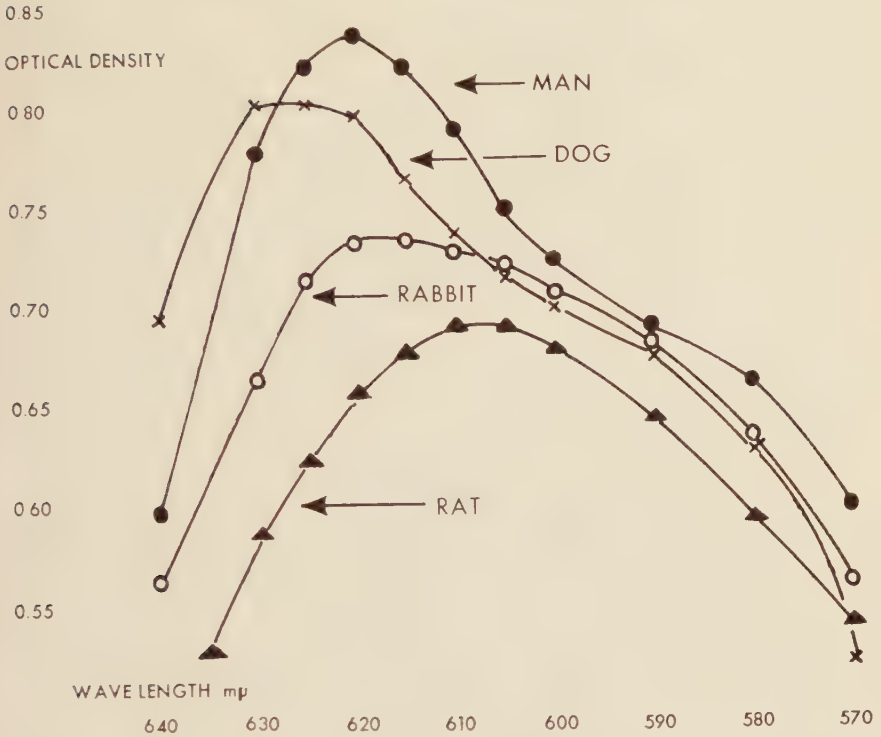

FIGURE 1. The absorption spectra of T-1824 in the plasma of man, the dog, the rabbit, and the rat. The optical density of 0.5-cm. thicknesses of 1:250 dilutions of 0.456 gm. per cent of T-1824 in the plasma of these animals is plotted. The values are taken from Allen, Ochoa, Roth, and Gregersen.[8]

and does this lead to "gobbling"? I think this must be the case. The rates of loss of T-1824 from the rabbit blood stream suggest a weaker binding, and the experiments of Allen and his co-workers[8] support this theory. In FIGURE 1 are 4 absorption spectra of complexes of T-1824 with the albumin of man, the dog, the rabbit, and the rat, as reported by these investigators. Note how much the rabbit albumin complex differs from that in man and in the dog. The rat complex seems even more different; perhaps we may suspect that T-1824 will give inaccurate measurements of plasma volume in the rat also. This remains to be tested.

As a result of the several simultaneous measurements of plasma volume with T-1824 and cell volume with labeled cells, it became evident that, if these measurements are correct, an accurate measurement of blood volume required the simultaneous measurement of cell and plasma volume. The later demonstration of the remarkable constancy of the ratio F_{cells},[17, 18, 44] showed, however, that in appropriate circumstances an accurate estimate of blood volume could be obtained from a measurement of either plasma volume or cell volume and the percentage of venous cells, provided the mean value for F_{cells} was known. Thus, in a resting man in whom

F_{cells} averages 0.91, total blood volume (B.V.) can be determined from the measured plasma volume (P.V.) and the measured percentage of venous cells (per cent *Hct*) from the equation:

$$\text{B.V.} = (\text{P.V.} \times 100)/(100 - \text{per cent } Hct \times 0.91),$$

or from the measured cell volume (C.V.) and the equation:

$$\text{B.V.} = (\text{C.V.} \times 100)/(\text{per cent } Hct \times 0.91).$$

The measurements of plasma volume with I^{131}-labeled albumins have confirmed these conclusions and lent added support to the values for F_{cells} obtained in man and in the splenectomized dog. In the rabbit, however, these measurements indicate that the previously observed value for F_{cells} was too low. It is of some interest to note that the revised value of 0.89 now falls very close to the values seen in man and in the splenectomized dog; this suggests the same proportionate distribution of cells and plasma in all three.

Conclusions

From this brief review it is seen that, to date, the chief contribution made by I^{131}-labeled proteins has been to confirm measurements of plasma volume made by other methods, and to show that, in the rabbit, the dye method gives erroneous estimates. However, the use of I^{131}-labeled proteins is still in its infancy. If satisfactory preparations that meet the requirements described earlier are used, and if precautions are taken to avoid errors, then these labeled proteins will provide much new information. They should be particularly valuable in measuring the plasma volumes of organs and tissues, as well as those of small animals. It will also be of interest to label proteins other than serum albumin and to measure their volumes of distribution.

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Discussion of the Paper

Magnus I. Gregersen (*College of Physicians and Surgeons, Columbia University, New York, N. Y.*): The tagging of proteins with I^{131} has come into prominence for good reasons. The papers and discussions in this monograph must disclose, even to the casual observer, the great possibilities that lie ahead. It is evident also that there is need for more fundamental work in order to reach correct interpretations of the data obtained in biological experiments. However, one cannot expect enthusiastic investigators to resist the temptation to exploit a promising new tool in medical and biological research until the tool has been studied exhaustively. As a matter of fact, some impulsiveness is probably productive of the incentive needed to go down and dig at the fundamentals, even though mistakes may result.

A similar situation developed many years ago after Keith and his co-workers[1] hit upon the use of certain vital dyes for measuring plasma volume. The dye method came into wide use for measurements of blood volume in

animals and in man long before the dye-protein bond was studied or the spectral absorption characteristic of the dyes were examined systematically. Some of this work, such as Keith's[2] studies on soldiers wounded during World War I, was actually first-rate, but for a number of years it was discounted, as were nearly all determinations of plasma and blood volume that utilized the dye method. How can this be explained? In retrospect, some of the reasons seem ludicrous. Partly, it was a matter of faulty colorimetric technique[3, 4] comparable to an inadequate counting of I^{131} and, partly, it was a failure to realize that the spectral absorption of these vital dyes is affected considerably by salts and proteins,[5] although Sörensen[6] actually had called attention to this many years earlier in connection with his studies of colorimetric determination of *p*H. The König-Martens spectrophotometer clarified these problems.[4, 5] Subsequently, the relation between the structure and the behavior of similar dyes was studied.[7] With electrophoresis and the ultracentrifuge, Rawson[8] showed that T-1824 is bound preferentially to plasma albumin. During World War II we demonstrated, from rates of disappearance, that certain concepts of hemorrhagic and traumatic shock were false and, with the aid of the carbon monoxide method, which itself came under scrutiny, we began to ascertain why cell-volume and plasma-volume methods gave different results in determinations of total blood volume.[9] Also during this period, as E. B. Reeve has emphasized, we were able to show that bovine albumin, bovine globulin, and the polysaccharide SHI gave the same volume distribution as albumin tagged with T-1824.[10] Additional indirect evidence against the concept that "gobbling" of T-1824 was a source of error in measuring plasma volume was provided by the studies with small and very large doses of dye.[11, 12] The so-called "cat effect" claimed by certain English workers[13] was clearly not present in the dog. However, as Reeve has shown, the rabbit is one species in which the "gobbling factor" may influence the determination with T-1824 and this, in fact, is not surprising in the light of the comparative physiological studies[14] showing marked differences in the spectral absorption curves of T-1824 in the plasma of different species. The dye-albumin bond in the rabbit is clearly less strong than in the dog or in man as shown by the simple experiment of mixing dye-tinged rabbit plasma with dog plasma, whereupon the dye is transferred to the dog albumin. The same phenomenon led to difficulties in measuring plasma volume after the injection of polyvinyl pyrolidone (PVP) in man, because it "steals" the T-1824 from the plasma and alters the spectral absorption curve of the dye.

These experiences illustrate some of the unanticipated difficulties that were encountered with the T-1824 label. This monograph on I^{131} will, I am sure, forestall many such difficulties and hasten more intensive study of the basic characteristic of this promising label.

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CONCLUDING REMARKS

By S. P. Masouredis

University of Pittsburgh Medical School and Central Blood Bank, Pittsburgh, Pa.

It must be obvious from the diverse scientific disciplines represented by the contributors to this monograph that I^{131} protein labeling is an extremely useful and versatile procedure. However, even this publication, because of limitations of space, cannot record all of the investigations that have been performed with I^{131}-labeled proteins.

The availability of a simple method for labeling molecules with the importance that proteins have in biological processes is bound to excite great interest and attention. The use of I^{131} as a protein label, however, implies that the protein molecule has been altered in that iodine has been introduced artificially into the native protein. The problem posed is not whether the protein has been changed—it obviously has been altered—but rather the extent or degree of this change and the effect of this labeling procedure on any proposed study. In the space allotted to me I should like briefly to reemphasize some of the factors that have been recognized as important in the interpretation of data obtained with I^{131} proteins.

An important consideration in the use of proteins labeled in this manner is the technique employed for iodination. It has become increasingly evident[1, 2] that differences in iodinating technique will yield proteins that behave in a different manner biologically. A cogent illustration of this problem is the trace-iodination of diphtheria toxin. This agent has been trace-iodinated by 2 different methods.[3] In one method, free iodine was liberated in an acid medium with nitrite[4] and, in the other, iodination was performed by using the exchange of I^{131} with triiodide.[5] The use of the nitrite-acid in 2 experiments yielded a product with only one-third to one-tenth the toxicity of the native toxin. The other technique of iodination yielded a labeled toxin with essentially the same toxicity as that of the native toxin. These results clearly show the need for defining the method of iodination and of characterizing the product obtained with each technique. It is conceivable that some of the discrepancies that have arisen in reconciling the results of different investigators may be due, in part, to the use of dissimilar products, resulting from variations in iodination.

Another problem that has been raised concerning the use of I^{131} proteins is that involved in the radiochemical effects occurring in I^{131} proteins. The decay of this radioisotope releases energy of a magnitude many times that involved in chemical reactions. This released energy manifests itself as recoil energy and energy liberated from the interaction of β and γ radiation with the solution, either with the protein molecules or those of the solvent. In addition to these effects, one must consider the effect on the protein molecule of the transmutation of the iodine atom to xenon following the decay of each I^{131} nucleus. Using simplifying assumptions, it can be shown that the recoil energies of the more energetic β and γ radiations of I^{131} are

about 2 to 6 eV.,[6] energies just barely within the range required to break chemical bonds. The effects of β and γ radiation are more difficult to evaluate and are dependent on many factors such as the volume of the solution, its composition, and the concentration of protein.

Trace I^{131}-labeled diphtheria toxin has been stored for a period of time sufficient to dissipate 98 to 99 per cent of the total radioactivity. The concentration of toxin was about 150 μg. of nitrogen per ml. in a volume of 0.2 ml. with about 0.1 to 0.7 μc. of I^{131}. Little or no toxicity was lost from the preparation, the toxicity of which was unaltered after iodination. On the other hand, the I^{131} toxin that was partially detoxified after iodination lost an additional 75 per cent of its toxicity following storage.[3] Even though diphtheria toxin is a labile protein, a properly labeled preparation of I^{131} showed no radiochemical or storage effects under these experimental conditions, whereas a partially detoxified I^{131} toxin underwent a marked loss of toxicity after storage. The loss of toxicity in the partially detoxified I^{131} toxin may be due to the effect of storage alone or to radiochemical effects. If complete destruction of the toxin molecules containing I^{131} occurs, either as a result of recoil effects or from the transmutation of iodine, less than 0.1 per cent of the diphtheria toxin would be inactivated under the conditions employed for these studies (specific activity of I^{131} and molar ratio of iodine to proteins). Significant radiochemical effects, if present in I^{131} proteins, are probably due to β-radiation effects.

At this point I should like to make a special plea concerning the presentation of results. Graphical analysis of semilogarithmic curves can be misleading. The number of components derived from such curves depends primarily on the imagination of the investigator. I do not know whether this difficulty can be controlled adequately, but there are some procedures that will minimize overreading of these curves. First, one can use the least-square method or some objective procedure to fit a curve to the experimental points and, second, it is relatively simple to determine the standard error of the slope of the regression line. Armed with this statistical information, half times and slopes then can be compared or treated with some degree of objectivity.

Finally, I shall comment upon the discrepancies between proteins labeled with I^{131} and those tagged with C^{14} or S^{35}. I do not know how to reconcile the findings that have been reported which, in some cases, show a difference and, in other cases, no difference between proteins labeled with these isotopes. Many possible explanations have been suggested by the contributors to this monograph. However, I wish to emphasize that, before one accepts as valid a difference between an iodine-labeled protein and a carbon- or sulfur-labeled protein, all of the factors affecting the iodinated protein must be evaluated critically.

It is clear that much work remains to be done with this problem, and that all of the answers are not available at this time. The iodination technique, however, is an extremely powerful tool in the hands of the investigator, and I feel confident that it can yield useful results so long as the investigator is aware of the limitations of the method.

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